jc690 U.S. PTO
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Anticipated Classification of this Application:	Prior Application:	Attorney's Docket
Class ___ Subclass ___	Examiner ___ Group Art Unit ___	No. 51320-AA/JPW/SHS

HONORABLE ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

November 28, 2000

jc520 U.S. PTO 09/724105 11/28/00

S I R:

This is a request for filing a X CONTINUATION ___ DIVISIONAL ___ CONTINUATION-IN-PART application under X 37 C.F.R. §1.53(b)[1] ___ 37 C.F.R. §1.53(d), of pending prior application Serial No. 08/874,618 filed on June 13, 1997 of Graham P. Allaway, et al. for
Inventor(s)

USES OF A CHEMOKINE RECEPTOR FOR INHIBITING HIV-1 INFECTION
Title of Invention

1. X Enclosed is a copy of the prior application, as originally filed and an affidavit or declaration verifying it as a true copy.

2. X A verified statement to establish small entity status under 37 C.F.R. §1.9 and 1.27

   ___ is enclosed.

   X was filed in the prior application and such status is still proper and desired (37 C.F.R. §1.28(a)).

3. X The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT

	NUMBER FILED		NUMBER EXTRA*		RATE SMALL ENTITY	RATE OTHER ENTITY		FEE SMALL ENTITY	FEE OTHER ENTITY
Total Claims	15-20	=	0	X	9	18	=	$ 0	$ 0
Independent Claims	11-3	=	8	X	40	80	=	$ 320	$ 0
Multiple Dependent Claims Presented: ___ Yes X No					135	270	=	$ 0	$ 0
					BASIC FEE			$ 355	$ 710
					TOTAL FEE			$675.00	$ 0

*If the difference in Col. 1 is less than zero, enter "0" in Col. 2.

[1] Filing an application pursuant to this section expressly abandons the parent application.

4. __x__ The Commissioner is hereby authorized to charge payment of the following fees associated with this application or credit any overpayment to Deposit Account No. 03-3125.

__x__ Any additional filing fees required under 37 C.F.R. §1.16.

__x__ Any patent application processing fees under 37 C.F.R. §1.17.

_____ The issue fees set forth in 37 C.F.R. §1.18 at or before mailing of the Notice of Allowance, pursuant to 37 C.F.R. §1.311(b).

5. __X__ Three copies of this sheet are enclosed.

6. __X__ A check in the amount of $ 675.00 is enclosed.

7. _____ Cancel claims ____________________.

8. _____ Amend the specification by inserting before the first line the sentence: --This is a ___continuation ___division of application Serial No.__________, filed __________.--

9. __X__ 12 Sheet(s) of ___ informal 10 formal drawing(s) is/are enclosed.

10. _____ Transfer the drawings from the prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file.

11. _____ Priority of application No. __________ filed on ____________________ in ____________________ (country) is claimed under 37 U.S.C. §119.

_____ The certified copy of the priority application has been filed in prior application Serial No. ______________, filed ______________.

12. __X__ The prior application is assigned of record to Progenics Pharmaceuticals, Inc. and Aaron Diamond AIDS Research Centre(ADARC)

13. __X__ A preliminary amendment is enclosed.

14. __X__ The power of attorney in the prior application is to:

John P. White (Reg. No. 28,678); Thomas F. Moran (Reg. No. 16,579); Norman H. Zivin (Reg. No. 25,385); Ivan S. Kavrukov (Reg. No. 25,161); Christopher C. Dunham (Reg. No. 22,031); Thomas G. Carulli (Reg. No. 30,616); Robert D. Katz (Reg. No. 30,141); Peter J. Phillips (Reg. No. 29,691); Richard S. Milner (Reg. No. 33,970); Albert Wai-Kit Chan (Reg. No. 36,479); Mary Anne P. Tanner (Reg. No. 40,197); and Mary Catherine DiNunzio (Reg. No. 37,306)

Applicants: Graham P. Allaway, et al.
Serial No.: Not Yet Known
Filing Date: November 28, 2000

(a) X The power appears in the original papers in the prior application.

(b) ____ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

(c) X Address all future communications to: (May only be completed by applicant, or attorney or agent of record.)

John P. White

Cooper & Dunham LLP

1185 Avenue of the Americas

New York, New York 10036

15. X Also enclosed Express Mail Certificate of Mailing No.

EE474771665US dated November 28, 2000

16. ____ I hereby verify that the attached papers are a true copy of prior application Serial No.__________ as originally filed on ______________________.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

November 28, 2000
Date

Signature John P. White
Reg. No. 28,678

___ INVENTOR(S)
___ ASSIGNEE OF COMPLETE INTEREST
X ATTORNEY OR AGENT OF RECORD
___ FILED UNDER 37 C.F.R. §1.34(a)

Address of Signator:

Cooper & Dunham LLP

1185 Avenue of the Americas

New York, New York 10036

Dkt. No. 51320-AA/JPW/SHS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Graham P. Allaway, et al.
Serial No.	:	Not Yet Known (continuation of U.S. Serial No. 08/874,618, filed June 13, 1997)
Filed	:	November 28, 2000
For	:	USES OF A CHEMOKINE RECEPTOR FOR INHIBITING HIV-1 INFECTION

1185 Avenue of the Americas
New York, New York 10036
November 28, 2000

Assistant Commissioner for Patents
Washington, D.C. 20231
Box: Patent Application

SIR:

PRELIMINARY AMENDMENT

Please amend the subject application as follows:

In the specification:

On page 1, line 1, after the words "This application is a" and before the words "claims priority of" please insert the following:

--continuation application of U.S. Serial No. 08/874,618, filed June 13, 1997, which--.

In the claims:

Please cancel claims 2-5, 7, 10, 12, 14-16, 18, 20-21, 23-25, 28-30, 32-35, 37-42 and 44-47 without prejudice or disclaimer to applicants' right to pursue the subject matter of these claims in a later-filed application. Please also amend claims 11, 26 and 48 as follows:

--11. (Amended) A method of treating an HIV-1 infected subject which comprises administering to the subject the polypeptide of [any of claims] claim 1[, 2, 3, 4,

6, or 7] in an amount effective to inhibit the fusion of HIV-1 to $CD4^{+}$ cells of the subject and thus treat the subject.--

--26. (Amended) A method for treating HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 19[, 20, 21, 22 or 23] to the subject.--

--48. A method for treating HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 42 [or 46] to the subject, so as to thereby treat HIV-1 infection in the subject.--

Remarks:

Claims 1-48 are pending in the subject application. Applicants have hereinabove canceled claims 2-5, 7, 10, 12, 14-16, 18, 20-21, 23-25, 28-30, 32-35, 37-42 and 44-47 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application and amended claims 11, 26, and 48. These amendments do not involve any issue of new matter. Accordingly, entry of this amendment is respectfully requested such that claims 1, 6, 8-9, 11, 13, 17, 19, 22, 26-27, 31, 36, 43 and 48 will be pending.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorneys invites the Examiner to telephone them at the number provided below.

No fee, in addition to the enclosed filing fee of $670.00 which includes the $355.00 filing fee and $320.00 fee for additional claims, is deemed necessary in connection with the filing of this Preliminary Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

John P. White
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Attorneys for Applicant(s)
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Dkt. No. 51320-A/JPW/AKC

Application
for
United States Letters Patent

To all whom it may concern:

Be it known that

Graham P. Allaway, Tatjana Dragic, Virginia M. Litwin, Paul J. Maddon, John P. Moore, and Alexandra Trkola

have invented certain new and useful improvements in

USES OF A CHEMOKINE RECEPTOR FOR INHIBITING HIV-1 INFECTION

of which the following is a full, clear and exact description.

Dkt. 51320-A/JPW/AKC

USES OF A CHEMOKINE RECEPTOR FOR INHIBITING HIV-1 INFECTION

This application claims priority of U.S. Provisional Application Serial No. 60/019,941, filed June 14, 1996, the content of which is incorporated into this application by reference.

The invention described in this application was made with support under Grants Nos. A135522, A136057, A136082 and A138573 from the National Institutes of Health, U.S. Department of Health and Human Service. Accordingly, the United States Government has certain rights in this invention.

Throughout this application, various references are referred to by arabic numerals within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each series of experiments.

Background of the Invention

The replication of primary, non-syncytium-inducing (NSI) HIV-1 isolates in $CD4^+$ T-cells is inhibited by the C-C ß-chemokines MIP-1α, MIP-1ß and RANTES (1,2), but T-cell line-adapted (TCLA) or syncytium-inducing (SI) primary strains are insensitive (2,3). The ß-chemokines are small (8kDa), related proteins active on cells of the lymphoid and monocyte lineage (4-8). Their receptors are members of the 7-membrane-spanning, G-protein-linked superfamily, one of which (the LESTR orphan receptor) has been identified as the second receptor for TCLA HIV-1 strains, and is now designated fusin (9). Fusin is not known to be a ß-chemokine receptor (7-9).

Summary of the Invention

This invention provides a polypeptide having a sequence corresponding to the sequence of a portion of a chemokine receptor capable of inhibiting the fusion of HIV-1 to $CD4^+$ cells and thus infection of the cells. In an embodiment, the chemokine receptor is C-C CKR-5. The CCKR-5 is also named as CCR5. In another embodiment, the polypeptide comprises amino acids having a sequence of at least one extracellular domain of C-C CKR-5.

In a preferred embodiment, the portion of a chemokine receptor comprises amino acid sequence MDYQVSSPIYDINYYTSEPCQKINVKQIAAR (SEQ ID NO: 5). In another preferred embodiment, the portion comprises amino acid sequence HYAAAQWDFGNTMCQ (SEQ ID NO: 6). In still another preferred embodiment, the portion comprises amino acid sequence RSQKEGLHYTCSSHFPYSQYQFWKNFQTLKIV (SEQ ID NO: 7). In a separate preferred embodiment, the portion comprises amino acid sequence QEFFGLNNCSSSNRLDQ (SEQ ID NO: 8). The portion of the chemokine receptor C-C CKR-5 may comprise one or more of the above sequences. The polypeptides may contain part of the above illustrated sequences and still be capable of inhibiting HIV-1 infection. The minimal number of amino acids sufficient to inhibit HIV-1 infection may be determined by the RET or infection assays as described below.

This invention also provides a pharmaceutical composition comprising effective amount of one or more of the above polypeptides and a pharmaceutically acceptable carrier.

This invention also provides a polypeptide having a sequence corresponding to that of a portion of an HIV-1 glycoprotein capable of specifically binding to the chemokine receptor C-C CKR-5.

This invention provides a pharmaceutical composition comprising effective amount of one of more polypeptides having a sequence corresponding to the sequence of a portion of an HIV-1 glycoprotein capable of specifically binding to the chemokine receptor C-C CKR-5 and a pharmaceutically acceptable carrier.

This invention provides an antibody or a portion of an antibody capable of binding to a chemokine receptor on a $CD4^+$ cell and inhibiting HIV-1 infection of the cell.

This invention also provides a pharmaceutical composition comprising an effective amount of an antibody capable of binding to a chemokine receptor on a $CD4^+$ cell and inhibiting HIV-1 infection of the cell and a pharmaceutically acceptable carrier.

This invention provides a method of treating an HIV-1 infected subject comprising administering to the subject the above polypeptides, antibody and pharmaceutical compositions.

This invention provides a method of reducing the likelihood of a subject from becoming infected by HIV-1 comprising administering to the subject the above pharmaceutical compositions.

This invention provides a method for inhibiting fusion of HIV-1 to $CD4^+$ cells which comprises contacting $CD4^+$ cells with a non-chemokine agent capable of binding to the chemokine receptor C-C CKR-5 in an amount and under conditions such that fusion of HIV-1 to the $CD4^+$ cells is inhibited.

This invention provides a method for inhibiting HIV-1 infection of $CD4^+$ cells which comprises contacting $CD4^+$ cells with a non-chemokine agent capable of binding to the chemokine receptor C-C CKR-5 in an amount and under conditions such that fusion of HIV-1 to the $CD4^+$ cells is inhibited, thereby inhibiting HIV-1 infection of the cells.

This invention provides a non-chemokine agent capable of binding to the chemokine receptor C-C CKR-5 and inhibiting HIV-1 infection.

This invention provides a molecule capable of binding to the chemokine receptor C-C CKR-5 and inhibiting HIV-1 infection comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the $CD4^+$ cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the other receptor.

This invention provides a pharmaceutical composition comprising an amount of the above molecules effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention also provides a molecule capable of binding to the chemokine receptor C-C CKR-5 and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent.

This invention further provides a pharmaceutical composition comprising an amount of the molecule capable of binding to the chemokine receptor C-C CKR-5 and inhibiting HIV-1 infection comprising a non-chemokine agent linked to a

compound capable of increasing the *in vivo* half-life of the non-chemokine agent effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention provides a method for reducing the likelihood of HIV-1 infection in a subject comprising administering the above pharmaceutical compositions to the subject.

This invention provides a method for treating HIV-1 infection in a subject comprising administering the above pharmaceutical compositions to the subject.

This invention provides a method for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a $CD4^+$, C-C $CKR-5^+$ cell which comprises: (a) contacting a $CD4^+$, C-C $CKR-5^+$ cell, which is labeled with a first dye, with a cell expressing an appropriate HIV-1 envelope glycoprotein on its surface, which is labeled with a second dye, in the presence of excess of the agent under conditions permitting the fusion of the $CD4^+$ and C-C $CKR-5^+$ cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of an agent known to inhibit fusion of HIV-1 to $CD4^+$, C-C $CKR-5^+$ cell, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and (c) determining whether there is resonance energy transfer, the absence or reduction of transfer indicating that the agent is capable of inhibiting fusion of HIV-1 to $CD4^+$ and C-C $CKR-5^+$ cells.

This invention also provides a transgenic nonhuman animal which comprises an isolated DNA molecule encoding the chemokine receptor C-C CKR-5. In an embodiment, this transgenic nonhuman animal further comprises an isolated DNA

molecule encoding a sufficient portion of the CD4 molecule to permit binding the HIV-1 envelope glycoprotein.

This invention further provides a transgenic nonhuman animal which comprises an isolated DNA molecule encoding the chemokine receptor C-C CKR-5 and an isolated DNA molecule encoding fusin. In an embodiment, this transgenic nonhuman animal further comprises an isolated DNA molecule encoding the full-length or portion of the CD4 molecule sufficient for binding the HIV-1 envelope glycoprotein.

This invention also provides transformed cells which comprise an isolated nucleic acid molecule encoding the chemokine receptor C-C CKR-5.

Finally, his invention provides an agent capable of inhibiting HIV-1 infection and capable of binding to a chemokine receptor without substantially affecting the said chemokine receptor's capability to bind to chemokines.

Description of the Figures

Figure 1: Specificity. time course and stage of ß-chemokine inhibition of HIV-1 replication

(1A) PM1 cells (1 x10^6) were preincubated with RANTES + MIP- 1α + MIP-1ß (R/Mα/Mß; 100ng/ml of each) for 24h (-24h) or 2h (-2h), then washed twice with phosphate buffered saline (PBS). HIV-1 (BaL env-complemented) virus (50ng of p24; see legend to Table 1) was added for 2h, then the cells were washed and incubated for 48h before measurement of luciferase activity in cell lysates as described previously (10,11). Alternatively, virus and R/Mα/Mß were added simultaneously to cells, and at the indicated time points (1h, 3h, etc) the cells were washed twice in PBS, resuspended in culture medium and incubated for 48h prior to luciferase assay. Time 0 represents the positive control, to which no ß-chemokines were added. +2h represents the mixture of virus with cells for 2h prior to washing twice in PBS, addition of R/Mα/Mß and continuation of the culture for a further 48h before luciferase assay.

(1B) PM1 cells (1x10^6) were infected with HIV-1 (500pg p24) grown in CEM cells (NL4/3; lanes 1-4) or macrophages (ADA; lanes 5-8), in the presence of 500ng/ml of RANTES (lanes 1 and 5) or MIP-1ß (lanes 2 and 6), or with no ß-chemokine (lanes 4 and 8). Lanes 3 and 7 are negative controls (no virus). All viral stocks used for the PCR assay were treated with DNAse for 30 min at 37°C, and tested for DNA contamination before use. After 2h, the cells were washed and resuspended in medium containing the same ß-chemokines for a

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further 8h. DNA was then extracted from infected cells using a DNA/RNA isolation kit (US Biochemicals). First round nested PCR was performed with primers: U3+, 5'-CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGG-3' (SEQ ID NO:1) preGag, 5'-AGCAAGCCGAGTCCTGCGTCGAGAG-3' (SEQ ID NO:2) and the second round with primers: LTR-test, 5'-GGGACTTTCCGCTGGGGACTTTC 3' (SEQ ID NO :3) LRC2, 5'-CCTGTTCGGGCGCCACTGCTAGAGATTTTCCAC 3' (SEQ ID NO:4) in a Perkin Elmer 2400 cycler with the following amplification cycles: 94°C for 5 min, 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 30s, 72°C for 7 min. M indicates 1kb DNA ladder; 1, 10, 100, 1000 indicate number of reference plasmid (pAD8) copies. The assay can detect 100 copies of reverse transcripts.

Figure 2: <u>HIV-1 env-mediated membrane fusion of cells transiently expressing C-C CKR-5</u>
Membrane fusion mediated by ß-chemokine receptors expressed in HeLa cells was demonstrated as follows: Cells were transfected with control plasmid pcDNA3.1 or plasmid pcDNA3.1 -CKR constructs using lipofectin (Gibco BRL). The pcDNA3.1 plasmid carries a T7-polymerase promoter and transient expression of ß-chemokine receptors was boosted by infecting cells with $1x10^7$ pfu of vaccinia encoding the T7-polymerase (vFT7.3) 4h post-lipofection (9). Cells were then cultured overnight in R18-containing media and were tested for their ability to fuse with HeLa-JR-FL cells (filled columns) or HeLa-BRU cells (hatched column) in the RET assay. The %RET with control HeLa cells was between 3% and 4% irrespective of the transfected plasmid.

Figure 3: <u>CD4-dependent competition between gp120 and MIP-1β for CCR-5 binding.</u>

(3A) JR-FL gp120 (filled squares), LAI gp120 (filled triangles), JR-FL-ΔV3 gp120 (open squares or LAI-ΔV3 gp120 (open trangles) was added to activated $CD4^+$ T cells and the extent of specific ^{125}I-MIP-1β binding determined. Data shown are the means of three independent experiments, each performed in duplicate.

(3B) JR-FL gp120 (2 $\mu g\ ml^{-1}$) and ^{125}I-MIP-1β (0.1 nM) were added to activated $CD4^+$ T cells in the presence of the monoclonal antibody Q4120 (filled circles) or sCD4 (filled squares) at the concentrations indicated. The extent of specific ^{125}I-MIP-1β binding was determined, and the percentage inhibition of the gp120 competitive effect was calculated for each antibody concentration (none present is 0% inhibition). The experiment shown was one of two performed, each yielding similar results.

Figure 4: <u>Mutagenesis of the predicted four extracellular domains of CCR5</u>

The amino acid sequences of the human CCR5 amino terminus (Nt) and three extracellular loops (ECL 1-3) are indicated(19,20 of the Third Series of Experiments). The polarity (+ or-) of charged residues is indicated below the main sequences, as are the identities of residues which differ in murine CCR5. Human CCR5 residues with negatively (white squares) and positively charged side chains (black squares), and residues whose charge differed in murine CCR5 (white circles), were all

modified to alanine by PCR or site-directed mutagenesis. Fidelity was confirmed by sequencing both strands of the entire CCR5 coding region. In some cases, double mutants K171A/E172A, K191A/N192A and R274A/D276A were made, to preserve the overall net charge of their domain. The Nt double and triple mutants D2A/D11A and D2A/D11A/E18A were based on initial results with single residue mutants.

Figure 5: <u>HIV-1 co-receptor function of CCR5 mutants</u> Substitutions in (A) negatively charged residues; (B) positively charged residues; (C) selected murine residues differing from the human sequence were tested for their effects on HIV-1 entry. U87MG-CD4 cells were transiently lipofected with CCR5 mutants, then infected with NLluc/ADA (dark hatched bars), NLluc/JR-FL (light hatched bars) or NLluc/DH123 (white bars) luciferase-expressing chimeric viruses(1,2 of the Third Series of Experiments). Luciferase activity (luc c.p.s.) was measured 72h post-infection(1,2) and standardized for lipofection efficiency and receptor expression levels. The co-receptor activity of each mutant designated on the x-axis is expressed as a percentage of the wild-type co-receptor activity (100%), and is the mean ±s.d. of three independent experiments each performed in quadruplicate. (*) indicates that the amino acid is also different in murine CCR5. Similar results (not shown) were obtained with SCL-1-CD4 cells.

Figure 6: <u>Membrane fusion activity of CCR5 Nt mutants</u> HeLa-CD4 cells were lipofected with the Nt mutants indicated (or the pcDNA3.1 negative control plasmid), and tested 48h later for their ability to fuse with HeLa cells expressing the JR-FL env gene (black bars)(1,18 of the Third Series of Experiments). The vFT7pol system was used to enhance co-receptor expression (hatched bars)(1,4,5,13 of the Third Series of Experiments). The extent of cell-cell fusion was determined using the RET assay(1.18 of the Third Series of Experiments). The % RET values shown are the means ±s.d. of three independent experiments, each performed in duplicate.

Figure 7: <u>Competition between gp120 and CCR5 MAb 2D7 for CCR5 binding</u>
HeLa cells co-transfected with CD4 and either wild-type or mutant CCR5, and infected with vFT7pol to enhance receptor expression, were pre-incubated with or without 10μg/ml gp120 (JR-FL)(7) before addition of 2ng/ml of the PE-labeled 2D7 MAb(23,24 of the Third Series of Experiments) and FACS analysis to determine mean fluorescence intensity (m.f.i.). Inhibition of 2D7-PE binding is presented as [1-(m.f.i. with gp120/m.f.i. without gp120)] x 100%, and is the mean ±s.d. of three independent experiments.

Figure 8. <u>Flow cytometric analysis of the binding of sCD4-gp120 complexes to (B)CCR5$^-$ and (B)CCR5$^+$ L1.2 cells, a murine pre-B lymphoma line</u>
Cells are incubated for 15 min. with equimolar (~100nM) mixtures of sCD4 and biotinylated HIV-1$_{JR-FL}$ gp120 and then stained with a streptavidin-

phycoerythrin conjugate, fixed with 2% paraformaldehyde, and analyzed by FACS. Cell number is plotted on the y-axis.

Figure 9. Inhibition of binding of HIV-1_{JR-FL} gp120, complexed with sCD4, to butyrate-treated L1.2 $CCR5^+$ cells

The inhibitors were the CC chemokines MIP-1β or RANTES at the concentrations indicated on the x axis.

Figire 10 Inhibition of HIV-1 envelope-mediated cell fusion by the bicyclam JM3100

The inhibition was measured using the RET assay, with the cell combinations indicated.

Detailed Description of the Invention

This invention provides a polypeptide having a sequence corresponding to the sequence of a portion of a chemokine receptor capable of inhibiting the fusion of HIV-1 to $CD4^+$ cells and thus infection of the cell. In an embodiment, the chemokine receptor is C-C CKR-5 (CCR5). In another embodiment, the fragment comprises at least one extracellular domain of the chemokine receptor C-C CKR-5. In a further embodiment, the extracellular domain is the second extracellular loop of CCR5.

In a separate embodiment, the chemokine receptor is CCR3 or CKR-2b(31,32).

The sequence of a portion of the chemokine receptor includes the original amino acids or modified amino acids from the receptor, their derivatives and analogues. Such sequence should retain the ability to inhibit HIV-1 infection. Sequences of fusin are also included.

In a preferred embodiment, the portion of a chemokine receptor comprises amino acid sequence MDYQVSSPIYDINYYTSEPCQKINVKQIAAR (SEQ ID NO: 5). In another preferred embodiment, the portion comprises amino acid sequence HYAAAQWDFGNTMCQ (SEQ ID NO: 6). In still another preferred embodiment, the portion comprises amino acid sequence RSQKEGLHYTCSSHFPYSQYQFWKNFQTLKIV (SEQ ID NO: 7). In a separate preferred embodiment, the portion comprises amino acid sequence QEFFGLNNCSSSNRLDQ (SEQ ID NO: 8). The portion of the chemokine receptor C-C CKR-5 may comprise one or more of the above sequences. The polypeptides may contain part of the above illustrated sequences and still be capable of inhibiting HIV-1 infection. The minimal number of amino acids sufficient to inhibit HIV-1 infection may be determined by the RET or infection assays as described

below.

The polypeptides described above may be fusion molecules such that the fragments are linked to other molecules. In an embodiment, the molecule is a CD4-based molecule. CD4-based molecules are known in the art and described in Allaway et al. (1996), Patent Cooperation Treaty Application No. PCT/US95/08805, publication no. WO 96/02575, the content of which is incorporated by reference into this application. In another embodiment, the polypeptide contains multiple units of one or more portions of a chemokine receptor. In a preferred embodiment, the polypeptide contains sequences corresponding to multiple units of one or more extracellular domains of the chemokine receptor C-C CKR-5.

This invention also provides a pharmaceutical composition comprising effective amount of the above polypeptide and a pharmaceutically acceptable carrier.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents.

This invention also provides a polypeptide having a sequence corresponding to that of a portion of an HIV-1 envelope glycoprotein capable of specifically binding to the chemoreceptor C-C CKR-5. Such a sequence may be identified by routine experiments. For example, overlapping synthetic peptides representing fragments of gp120 or gp41 can be tested in the RET assay for their ability to inhibit fusion between cells expressing the envelope glycoprotein of HIV-1 clinical isolates and cells expressing CD4 and C-C CKR-5. Peptides inhibiting fusion in this assay are also screened

in the RET assay for ability to inhibit fusion mediated by the envelope glycoprotein of HIV-1 laboratory-adapted-strains and peptides which are inhibitory in this later assay are discarded. As an alternative method, the peptides can be tested for their ability to compete with chemokines for binding to cell expressing C-C CKR-5.

This invention provides a pharmaceutical composition comprising effective amount of the polypeptide comprising a fragment of HIV-1 glycoprotein capable of specifically binding to the chemokine receptor C-C CKR-5 and a pharmaceutically acceptable carrier.

This invention provides an antibody or a portion of an antibody thereof capable of binding to a chemokine receptor on a $CD4^+$ cell and inhibiting HIV-1 infection of the cell.

This invention also provides a pharmaceutical composition comprising effective amount of antibody capable of binding to a chemokine receptor and inhibiting HIV-1 infection and a pharmaceutically acceptable carrier.

This invention provides a method of treating an HIV-1 infected subject comprising administering to the subject the above pharmaceutical compositions.

This invention provides a method of reducing the likelihood of a subject from becoming infected by HIV-1 comprising administering to the subject the above pharmaceutical compositions.

This invention provides a method for inhibiting fusion of HIV-1 to $CD4^+$ cells which comprises contacting $CD4^+$ cells with a non-chemokine agent capable of binding to the chemokine receptor C-C CKR-5 in an amount and under

conditions such that fusion of HIV-1 to the $CD4^+$ cells is inhibited.

This invention provides a method for inhibiting HIV-1 infection of $CD4^+$ cells which comprises contacting $CD4^+$ cells with a non-chemokine agent capable of binding to the chemokine receptor C-C CKR-5 in an amount and under conditions such that fusion of HIV-1 to the $CD4^+$ cells is inhibited, thereby inhibiting HIV-1 infection.

The non-chemokine agents of this invention are capable of binding to chemokine receptors and inhibiting fusion of HIV-1 to $CD4^+$ cells. The non-chemokine agents include, but are not limited to, chemokine fragments and chemokine derivatives and analogues, but do not include naturally occurring chemokines.

In an embodiment, the non-chemokine agent is an oligopeptide. In another embodiment, the non-chemokine agent is a polypeptide. In still another embodiment, the non-chemokine agent is a nonpeptidyl agent.

This invention provides a non-chemokine agent capable of binding to the chemokine receptor C-C CKR-5 and inhibiting fusion of HIV-1 to $CD4^+$ cells.

This invention provides a molecule capable of binding to the chemokine receptor C-C CKR-5 and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the $CD4^+$ cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the other receptor. In an embodiment, the cell surface receptor is CD4. In another embodiment, the ligand comprises an

antibody or a portion of an antibody.

This invention provides a pharmaceutical composition comprising an amount of the above molecule effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention also provides a molecule capable of binding to the chemokine receptor C-C CKR-5 and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent. In an embodiment, the compound is polyethylene glycol.

This invention further provides a pharmaceutical composition comprising an amount of the molecule capable of binding to the chemokine receptor C-C CKR-5 and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention provides a method for reducing the likelihood of HIV-1 infection in a subject comprising administering the above pharmaceutical compositions to the subject.

This invention provides a method for treating HIV-1 infection in a subject comprising administering the above pharmaceutical compositions to the subject.

This invention provides a method for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a $CD4^+$, C-C CKR-5 $^+$ cell which comprises: (a) contacting a $CD4^+$, C-C CKR-5^+ cell, which is labeled with a first dye, with a cell expressing an appropriate HIV-1

envelope glycoprotein on its surface, which is labeled with a second dye, in the presence of excess of the agent under conditions permitting the fusion of the CD4$^+$ and C-C CKR-5$^+$ cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of an agent known to inhibit fusion of HIV-1 to CD4$^+$, C-C CKR-5$^+$ cell, the first and second dyes being selected so as to allow resonance energy transfer between the dyes; (b) exposing the product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and (c) determining whether there is resonance energy transfer, the absence or reduction of transfer indicating that the agent is capable of inhibiting fusion of HIV-1 to CD4$^+$ and C-C CKR-5$^+$ cells. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is a polypeptide. In still another embodiment, the agent is a nonpeptidyl agent. In a further embodiment, the CD4$^+$ cell is a PM1 cell. In a separate embodiment, the cell expressing the HIV-1 envelope glycoprotein is a HeLa cell expressing HIV-1_{JR-FL} gp120/gp41.

This invention also provides a transgenic nonhuman animal which comprises an isolated DNA molecule encoding the chemokine receptor C-C CKR-5. In an embodiment, this transgenic nonhuman animal further comprises an isolated DNA molecule encoding a sufficient portion of the CD4 molecule to permit binding the HIV-1 envelope glycoprotein.

This invention further provides a transgenic nonhuman animal which comprises an isolated DNA molecule encoding the chemokine receptor C-C CKR-5 and an isolated DNA molecule encoding fusin. In an embodiment, this transgenic nonhuman animal further comprises an isolated DNA molecule encoding a sufficient portion of the CD4 molecule to permit binding the HIV-1 envelope glycoprotein.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium (Hogan B. et al. Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)). DNA or cDNA encoding the C-C CKR-5 chemokine receptor or CD4 is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

This invention provides transformed cells which comprise an isolated nucleic acid molecule encoding the chemokine receptor C-C CKR-5.

This invention also provides an agent capable of inhibiting HIV-1 infection and capable of binding to a chemokine receptor without substantially affecting the said chemokine receptor's capability to bind to chemokines.

As used herein, the term "without substantially affecting" mean that after the binding of the agent to the chemokine receptor, the chemokine receptor should still be able to bind to chemokines. Under some conditions, following binding of an agent to a chemokine receptor, a higher concentration of the chemokine is required to achieve the degree of binding observed if the chemokine receptor had not been bound to the agent. In a preferred embodiment of this agent, the chemokine concentration required to achieve the same binding is two fold. In another embodiment, the chemokine concentration is ten fold.

In a preferred embodiment of this invention, the chemokine receptor is CCR5. In another embodiment, the chemokine receptor is CXCR4, CCR3 or CCR-2b.

This agent may be an oligopeptide, a nonpeptidyl agent or a polypeptide. Alternatively, this agent can be an antibody or a portion of an antibody.

This invention further provides a pharmaceutical composition comprising an amount of the above agent effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

This invention provides a method for inhibiting HIV-1 infection of $CD4^+$ cells which comprises contacting such $CD4^+$ cells with an agent capable of inhibiting HIV-1 infection and capable of binding to a chemokine receptor without substantially affecting the said chemokine receptor's capability to bind to chemokines.

This invention also provides a molecule capable of binding to the chemokine receptor CCR5 and inhibiting fusion of HIV-1 to $CD4^+$ cells without substantially affecting the said

chemokine receptor's capability to bind to chemokines linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent. In an embodiment, the compound is polyethylene glycol.

This invention provides a pharmaceutical composition comprising an amount of the above molecule effective to inhibit HIV-1 infection and a pharmaceutically acceptable carrier.

This invention provides a method for reducing the likelihood of HIV-1 infection in a subject comprising administering the above pharmaceutical composition to the subject.

This invention provides a method for treating HIV-1 infection in a subject comprising administering the above pharmaceutical composition to the subject.

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a chemokine receptor on a solid matrix; (b) contacting the agent with the fixed chemokine receptor under conditions permitting the binding of the agent to the chemokine receptor; (c) removing the unbound agent; (d) contacting the fixed chemokine receptor resulting in step (c) with a gp120 in the presence of CD4 under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (e) measuring the amount of bound gp120/CD4 complex; and (f) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention also provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a chemokine receptor on a solid matrix; (b) contacting the agent with the fixed chemokine receptor; (c) contacting the mixture in step (b) with a gp120 in the presence of CD4 under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (d) measuring the amount of bound gp120/CD4 complex; and (e) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention also provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a gp120/CD4 complex on a solid matrix; (b) contacting the agent with the fixed gp120/CD4 complex under conditions permitting the binding of the agent to the gp120/CD4 complex; (c) removing unbound agent;(d) contacting the fixed gp120/CD4 complex resulting from step (c) with a chemokine receptor under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (e) measuring the amount of bound chemokine receptor; and (f) comparing the amount determined in step (e) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

This invention provides a method for determining whether an agent is capable of inhibiting HIV-1 infection comprising steps of: (a) fixing a gp120/CD4 on a solid matrix; (b) contacting the agent with the fixed gp120/CD4 complex; (c) contacting the mixture in step (b) with a chemokine receptor

under conditions permitting the binding of the gp120/CD4 complex and the chemokine receptor in the absence of the agent; (d) measuring the amount of bound chemokine receptor; (e) comparing the amount determined in step (d) with the amount determined in the absence of the agent, a decrease of the amount indicating that the agent is capable of inhibiting HIV-1 infection.

As used in these assays, CD4 include soluble CD4, fragment of CD4 or polypeptides incorporating the gp120 binding site of CD4 capable of binding gp120 and enabling the binding of gp120 to the appropriate chemokine receptor.

As used in these assays, gp120 is the gp120 from an appropriate strain of HIV-1. For example, gp120 from the macrophage tropic clinical isolate HIV-1_{JR-FL} will bind to the chemokine receptor CCR5, whereas gp120 from the laboratory adapted T-tropic strain HIV-1_{LAI} will bind to the chemokine receptor CXCR4.

In a preferred embodiment of the above methods, the CD4 is a soluble CD4. The chemokine receptor which may be used in the above assay includes CCR5, CXCR4, CCR3 and CCR-2b.

In an embodiment, the chemokine receptor is expressed on a cell. In a preferred embodiment, the cell is a L1.2 cell. In a separate embodiment, the chemokine receptor is purified and reconstituted in liposomes. Such chemokine receptor embedded in the lipid bilayer of liposomes retains the gp120 binding activity of the receptor.

The gp120, CD4 or both may be labelled with a detectable marker in the above assays. Markers including radioisotope or enzymes such as horse radish peroxidase may be used in this invention.

In an embodiment, the gp120 or CD4 or the chemokine receptor is labelled with biotin. In a further embodiment, the biotinylated gp120, or CD4 or the chemokine receptor is detected by: (i) incubating with streptavidin-phycoerythrin, (ii) washing the incubated mixture resulting from step (i), and (iii) measuring the amount of bound gp120 using a plate reader, exciting at 530nm, reading emission at 590nm.

This invention also provides an agent determined to be capable of inhibiting HIV-1 infection by the above methods, which is previously unknown.

This invention also provides a pharmaceutical composition comprising the agent determined to be capable of inhibiting HIV-1 infection by the above methods and a pharmaceutically acceptable carrier. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is a polypeptide. In a still another embodiment, the agent is a nonpeptidyl agent.

This invention also provides a molecule capable of binding to the chemokine receptor CCR5 and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising the above determined agent linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent. In an embodiment, the compound is polyethylene glycol. This invention also provides a pharmaceutical composition comprising an amount of the above molecule effective to inhibit HIV-1 infection and a pharmaceutically acceptable carrier.

This invention provides a method for reducing the likelihood of HIV-1 infection in a subject comprising administering the above pharmaceutical compositions to the subject.

This invention provides a method for treating HIV-1 infection in a subject comprising administering the above pharmaceutical composition to the subject.

The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the invention as described herein, which is defined by the claims which follow thereafter.

Experimental Details

To study how ß-chemokines inhibit HIV-1 replication, a virus entry assay based on single-cycle infection by an env-deficient virus, NL4/3Δenv (which also carries the luciferase reporter gene), complemented by envelope glycoproteins expressed in trans was used (10,11). Various env-complemented viruses were tested in PM1 cells, a variant of HUT-78 that has the unique ability to support replication of primary and TCLA HIV-1 strains, allowing comparison of envelope glycoprotein functions against a common cellular background (2,12). MIP-1α, MIP-1ß and RANTES are most active against HIV-1 in combination (2,3), and strongly inhibited infection of PM1 cells by complemented viruses whose envelopes are derived from the NSI primary strains ADA and BaL (Table 1a).

Table 1: Inhibition of HIV-1 entry in PM1 cells and $CD4^+$ T-cells by ß-chemokines

% luciferase activity

	BaL	ADA	NL4/3	HxB2	MuLV
a)					
PM1 cells					
control without virus	2	2	2	5	3
control with virus	100	100	100	100	100
+R/Mα/Mß (50/50/50)	2	3	92	117	100
+RANTES (100)	1	1	nd	nd	nd
+MIP-1α(100)	54	54	nd	nd	nd
+MIP-1ß (100)	1	6	nd	nd	nd
+MCP-1 (100)	46	50	nd	nd	nd
+MCP-2 (100)	28	26	nd	nd	nd
+MCP-3 (100)	58	46	nd	nd	nd
b)	**JR-FL**	**HxB2**	**MuLV**		
LW4 $CD4^+$ T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	14	68	nd		
LW5 $CD4^+$ T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	15	73	nd		

Table 1 legend:

PM1 cells were cultured as described by Lusso et al (12). Ficoll/hypaque-isolated PBMC from laboratory workers (LW) stimulated with PHA for 72h before depletion of CD8+ Lymphocytes with anti-CD8 immunomagnetic beads (DYNAL, Great Neck, NY). CD4+ Lymphocytes were maintained in culture medium containing interleukin-2 (100U/ml; Hofmann LaRoche, Nutley, NJ), as described previously (3). Target cells (1-2x10^5) were infected with supernatants (10-50ng of HIV-1 p24) from 293-cells co-transfected with an NL4/3Δenv-luciferase vector and a HIV-1 env-expressing vector (10,11). ß-Chemokines (R & D Systems, Minneapolis) were added to the target cells simultaneously with virus, at the final concentrations (ng/ml) indicated in parentheses in the first column. The ß-chemokine concentration range was selected based on prior studies (2,3). After 2h, the cells were washed twice with PBS, resuspended in ß-chemokine-containing media and maintained for 48-96h. Luciferase activity in cell lysates was measured as described previously (10,11). The values indicated represent luciferase activity (cpm)/ng p24/mg protein, expressed relative to that in virus-control cultures lacking ß-chemokines (100%), and are the means of duplicate or sextuplicate determinations. nd, not done. R/Mα/Mß, RANTES + MIP-1α + MIP-1ß.

RANTES and MIP-1ß were strongly active when added individually, while other ß-chemokines - MlP-1α, MCP-1, MCP-2 and MCP-3 (refs. 13-15) - were weaker inhibitors (Table 1a). However, MIP-1α, MIP-1ß and RANTES, in combination, did not inhibit infection of PM1 cells by the TCLA strains NL4/3 and HxB2, or by the amphotropic murine leukemia virus (MuLV-Ampho) pseudotype (Table 1a). Thus,

phenotypic characteristics of the HIV-1 envelope glycoproteins influence their sensitivity to ß-chemokines in a virus entry assay.

The *env*-complementation assay was used to assess HIV-1 entry into CD4+ T-cells from two control individuals (LW4 and LW5). MIP-1α, MIP-1ß and RANTES strongly inhibited infection by the NSI primary strain JR-FL infection of LW4's and LW5's $CD4^+$ T-cells, and weakly reduced HxB2 infection of LW cells (Table 1b), suggesting that there may be some overlap in receptor usage on activated $CD4^+$ T-cells by different virus strains. BaL env-mediated replication in normal PBL was also inhibited by MIP-1α, MIP-1ß and RANTES, albeit with significant inter-donor variation in sensitivity (data not shown).

It was determined when ß-chemokines inhibited HIV-1 replication by showing that complete inhibition of infection of PM1 cells required the continuous presence of ß-chemokines for up to 5h after addition of ADA or BaL env-complemented virus (Fig.1a). Pre-treatment of the cells with ß-chemokines for 2h or 24h prior to infection had no inhibitory effect if the cells were subsequently washed before virus addition. Furthermore, adding ß-chemokines 2h after virus only minimally affected virus entry (Fig.1a). A PCR-based assay was next used to detect HIV-1 early DNA reverse transcripts in PM1 cells after 10h of infection; reverse transcription of ADA, but not of NL4/3, could not be detected in the presence of MIP-1ß and RANTES (Fig.1b). Thus, inhibition by ß-chemokines requires their presence during at least one of the early stages of HIV-1 replication: virus attachment, fusion and early reverse transcription.

These sites of action were discriminated, first by testing whether ß-chemokines inhibited binding of JR-FL or BRU gp120 to soluble CD4, or of tetrameric CD4-IgG2 binding to HeLa-JR-FL cells expressing oligomeric envelope glycoproteins (17). No inhibition by any of the ß-chemokines was found in either assay, whereas the OKT4a CD4-MAb was strongly inhibitory in both (data not shown). Thus, ß-chemokines inhibit a step after CD4 binding, when conformational changes in the envelope glycoproteins lead to fusion of the viral and cellular membranes (18). Cell-cell membrane fusion is also induced by the gp120-CD4 interaction, and can be monitored directly by resonance energy transfer (RET) between fluorescent dyes incorporated into cell membranes (17). In the RET assay, OKT4a completely inhibits membrane fusion of PM1 cells with HeLa cells expressing the envelope glycoproteins of either JR-FL (HeLa-JR-FL) or BRU (HeLa-BRU), confirming the specificity of the process (17). RANTES, MIP-1ß (and to a lesser extent, MIP-1α) strongly inhibited membrane fusion of HeLa-JR-FL cells with PM1 cells, whereas fusion between PM1 cells and HeLa-BRU cells was insensitive to these ß-chemokines (Table 2a).

Table 2: Effect of ß-chemokines on HIV-1 envelope glycoprotein-mediated membrane fusion measured using the RET assay

	% Fusion HeLa-JR-FL	HeLa-BRU
a) <u>PM1 cells</u>		
no chemokines	100	100
+R/Mα/Mß (80/400/100)	1	95
+RANTES (80)	8	100
+MIP-1α (400)	39	100
+MIP-1ß (100)	13	93
+MCP-1 (100)	99	98
+MCP-2 (100)	72	93
+MCP-3 (100)	98	99
b) <u>LW5 CD4+ cells</u>		
no chemokines	100	100
+R/Mα/Mß(106/533/133)	39	100
+RANTES (106)	65	95
+MIP-1α (533)	72	100
+MIP-1ß (133)	44	92
+OKT4A (3ug/ml)	0	0

Table 2 legend:
$CD4^+$ target cells (mitogen-activated $CD4^+$ lymphocytes or PM1 cells) were labeled with octadecyl rhodamine (Molecular Probes, Eugene, OR), and HeLa-JR-FL cells, HeLa-BRU cells (or control HeLa cells, not shown) were labeled with octadecyl fluorescein (Molecular Probes), overnight at 37°C. Equal numbers of labeled target cells and env-expressing cells were mixed in 96-well plates and ß-chemokines (or CD4 MAb OKT4a) were added at the final concentrations (ng/ml) indicated in parentheses in the first column. Fluorescence emission values were determined 4h after cell mixing (17). If cell fusion occurs, the dyes are closely associated in the conjoined membrane such that excitation of fluorescein at 450nm results in resonance energy transfer (RET) and emission by rhodamine at 590nm. Percentage fusion is defined as equal to 100 x [(Exp RET - Min RET) / (Max RET - Min RET)], where Max RET = %RET obtained when HeLa-Env and $CD4^+$ cells are mixed, Exp RET = %RET obtained when HeLa-Env and $CD4^+$ cells are mixed in the presence of fusion-inhibitory compounds, and Min RET = %RET obtained when HeLa cells (lacking HIV-1 envelope glycoproteins) and $CD4^+$ cells are mixed. The %RET value is defined by a calculation described elsewhere(17), and each is the mean of triplicate determinations. These values were, for HeLa-JR-FL and HeLa-BRU cells respectively: PM1 cells 11.5%, 10.5%; LW5 $CD4^+$ cells, 6.0%, 10.5%; R/Mα/Mß, RANTES + MIP-1α + MIP-1ß.

Similar results were obtained with primary $CD4^+$ T-cells from LW5 (Table 2b), although higher concentrations of ß-chemokines were required to inhibit membrane fusion in the primary cells than in PM1 cells. Thus, the actions of the ß-chemokines are not restricted to the PM1 cell line. The RET assay demonstrates that ß-chemokines interfere with env-mediated membrane fusion.

The simplest explanation of these results is that the binding of certain ß-chemokines to their receptor(s) prevents, directly or otherwise, the fusion of HIV-1 with CD4+ T-cells. It has been known for a decade that HIV-1 requires a second receptor for entry into CD4+ cells (19-21). This function is supplied, for TCLA strains, by fusin (9). Several receptors for MIP-1α, MIP-1ß and RANTES have been identified (6,7), and ß-chemokines exhibit considerable cross-reactivity in receptor usage (4-8). However, C-C CKR-1 and, especially, C-C CKR-5 were identified as the most likely candidates, based on tissue expression patterns and their abilities to bind MIP-1α, MIP-1ß and RANTES (4,7,8,15,22). C-C CKR-1, C-C CKR-5 and LESTR are each expressed at the mRNA level in PM1 cells and primary macrophages (data not shown). These and other ß-chemokine receptors were therefore PCR-amplified, cloned and expressed.

The expression of C-C CKR-5 in HeLa-CD4 (human), COS-CD4 (simian) and 3T3-CD4 (murine) cells rendered each of them readily infectible by the primary, NSI strains ADA and BaL in the env-complementation assay of HIV-1 entry (Table 3).

Table 3: C-C CKR-5 expression permits infection of CD4-expressing cells by primary, NSI HIV-1 strains

									R/Mα/Mß
		pcDNA3.1	LESTR	CKR-1	CKR-2a	CKR-3	CKR-4	CKR-5	CKR-5
COS-CD4	ADA	798	456	600	816	516	534	153000	3210
	BaL	660	378	600	636	516	618	58800	756
	HxB2	5800	96700	5240	5070	5470	5620	4850	5000
HeLa-CD4	ADA	678	558	4500	912	558	600	310000	6336
	BaL	630	738	1800	654	516	636	104000	750
	HxB2	337000	nd	nd	nd	nd	nd	nd	356000
3T3-CD4	ADA	468	558	450	618	534	606	28400	1220
	BaL	606	738	660	738	534	558	11700	756
	HxB2	456	24800	618	672	732	606	618	606

Table 3 legend:

Chemokine receptor genes C-C CKR-1, C-C CKR-2a, C-C CKR-3, C-C CKR-4 and C-C CKR-5 have no introns (4-8,15,22) and were isolated by PCR performed directly on a human genomic DNA pool derived from the PBMC of seven healthy donors. Oligonucleotides overlapping the ATG and the stop codons and containing BamHI and XhoI restriction sites for directional cloning into the pcDNA3.1 expression vector (Invitrogen Inc.) were used. LESTR (also known as fusin or HUMSTR) (4,9,24) was cloned by PCR performed directly on cDNA derived from PM1 cells, using sequences derived from the NIH database. Listed below are the 5'and 3' primer pairs used in first (5-1 and 3-1) and second (5-2 and 3-2) round PCR amplification of the CKR genes directly from human genomic DNA, and of LESTR from PM1 cDNA. Only a single set of primers was used to amplify CKR-5.

LESTR: L/5-1 = AAG CTT GGA GAA CCA GCG GTT ACC ATG GAG GGG ATC (SEQ ID NO: 9);

L/5-2 = GTC TGA GTC TGA GTC AAG CTT GGA GAA CCA (SEQ ID NO: 10);

L/3-1 = CTC GAG CAT CTG TGT TAG CTG GAG TGA AAA CTT GAA GAC TC (SEQ ID NO: 11);

L/3-2 = GTC TGA GTC TGA GTC CTC GAG CAT CTG TGT (SEQ ID NO: 12);

CKR-1:C1/5-1 = AAG CTT CAG AGA GAA GCC GGG ATG GAA ACT CC (SEQ ID NO: 13);

C1/5-2 = GTC TGA GTC TGA GTC AAG CTT CAG AGA GAA (SEQ ID NO: 14);

C1/3-1 = CTC GAG CTG AGT CAG AAC CCA GCA GAG AGT TC (SEQ ID NO: 15);

C1/3-2 = GTC TGA GTC TGA GTC CTC GAG CTG AGT CAG (SEQ ID NO: 16);

CKR-2a:C2/5-1 = AAG CTT CAG TAC ATC CAC AAC ATG CTG TCC AC (SEQ ID NO: 17);

C2/5-2= GTC TGA GTC TGA GTC AAG CTT CAG TAC ATC (SEQ ID NO: 18);

C2/3-1 = CTC GAG CCT CGT TTT ATA AAC CAG CCG AGA C (SEQ ID NO: 19);

C2/3-2 = GTC TGA GTC TGA GTC CTC GAG CCT CGT TTT (SEQ ID NO: 20);

CKR-3: C3/5-1 = AAG CTT CAG GGA GAA GTG AAA TGA CAA CC (SEQ ID NO: 21);

C3/5-2= GTC TGA GTC TGA GTC AAG CTT CAG GGA GAA (SEQ ID NO: 22);

C3/3-1 = CTC GAG CAG ACC TAA AAC ACA ATA GAG AGT TCC (SEQ ID NO: 23);

C3/3-2 = GTC TGA GTC TGA GTC CTC GAG CAG ACC TAA (SEQ ID NO: 24);

CKR-4: C4/5-1 = AAG CTT CTG TAG AGT TAA AAA ATG AAC CCC ACG G (SEQ ID NO: 25);

C4/5-2 = GTC TGA GTC TGA GTC AAG CTT CTG TAG AGT (SEQ ID NO: 26);

C4/3-1 = CTC GAG CCA TTT CAT TTT TCT ACA GGA CAG CAT C (SEQ ID NO: 27);

C4/3-2 = GTC TGA GTC TGA GTC CTC GAG CCA TTT CAT (SEQ ID NO: 28);

CKR-5: C5/5-12 = GTC TGA GTC TGA GTC AAG CTT AAC AAG ATG GAT TAT CAA (SEQ ID NO: 29);

C5/3-12 = GTC TGA GTC TGA GTC CTC GAG TCC GTG TCA CAA GCC CAC (SEQ ID NO: 30).

The human CD4-expressing cell lines HeLa-CD4 (P42), 3T3-CD4 (sc6) and COS-CD4 (Z28T1) (23) were transfected with the different pcDNA3.1-CKR constructs by the calcium phosphate method, then infected 48h later with different reporter viruses (200ng of HIV-1 p24/10^6 cells) in the presence or absence of ß-chemokines (400ng/ml each of RANTES, MIP-1α and MIP-1ß). Luciferase activity in cell lysates was measured 48h later (10,11). ß-Chemokine blocking data is only shown for C-C CKR-5, as infection mediated by the other C-C CKR

genes was too weak for inhibition to be quantifiable. In PCR-based assays of HIV-1 entry, a low level of entry of NL4/3 and ADA into C-C CKR-1 expressing cells (data not shown) was consistently observed.

Neither LESTR nor C-C CKR-1, -2a, -3 or -4 could substitute for C-C CKR-5 in this assay. The expression of LESTR in COS-CD4 and 3T3-CD4 cells permitted HxB2 entry, and HxB2 readily entered untransfected (or control plasmid-transfected) HeLa-CD4 cells (Table 3). Entry of BAL and ADA into all three C-C CKR-5-expressing cell lines was almost completely inhibited by the combination of MIP-1α, MIP-1ß and RANTES, whereas HxB2 entry into LESTR-expressing cells was insensitive to ß chemokines (Table 3). These results suggest that C-C CKR-5 functions as a ß-chemokine-sensitive second receptor for primary, NSI HIV-1 strains.

The second receptor function of C-C CKR-5 was confirmed in assays of env-mediated membrane fusion. When C-C CKR-5 was transiently expressed in COS and HeLa cell lines that permanently expressed human CD4, both cell lines fused strongly with HeLa cells expressing the JR-FL envelope glycoproteins, whereas no fusion occurred when control plasmids were used (data not shown). Expression of LESTR instead of C-C CKR-5 did not permit either COS-CD4 or HeLa-CD4 cells to fuse with HeLa-JR-FL cells, but did allow fusion between COS-CD4 cells and HeLa-BRU cells (data not shown).

The fusion capacity of ß-chemokine receptors was also tested in the RET assay. The expression of C-C CKR-5, but not of C-C CKR-1, -2a, -3 or -4, permitted strong fusion between HeLa-CD4 cells and HeLa-JR-FL cells. The extent of fusion between HeLa-JR-FL cells and C-C CKR-5-expressing HeLa-CD4

cells was greater than the constitutive level of fusion between HeLa-BRU cells and HeLa-CD4 cells (Fig.2). The fusion-conferring function of C-C CKR-5 for primary, NSI HIV-1 strains has therefore been confirmed in two independent fusion assays.

Experimental Discussion

Together, the above results establish that MlP-1α, MIP-1ß and RANTES inhibit HIV-1 infection at the entry stage, by interfering with the virus-cell fusion reaction subsequent to CD4 binding. It was also shown that C-C CKR-5 can serve as a second receptor for entry of primary NSI strains of HIV-1 into CD4+ T-cells, and that the interaction of ß-chemokines with C-C CKR-5 inhibits the HIV-1 fusion reaction.

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Second Series of Experiments

Experimental Methods

Sources of reagents. Recombinant human ß-chemokines were purchased from R&D Systems (Minneapolis) and ^{125}I-MIP-1ß (specific activity 2,200 Ci mmol^{-1}) was from Dupont-NEN. Anti-CD4 monoclonal antibodies were from Q. Sattentau(16), except for 5A8, from L. Burkly (Biogen)(27) and L120 (UK MRC AIDS Reagent Repository)(16). Soluble CD4 has been described(17). Monoclonal antibodies to gp120 were obtained from donors listed elsewhere(19,20), except for 23A (gp120 C terminus, from J. Robinson), 447-D and 697-D (ref. 28; Cellular Products Inc.) And 83.1 (ref. 29; Repligen). Recombinant gp41 (IIIB) ectodomain was from Viral Therapeutics Inc., and V3 peptides were obtained either from Repligen or the UK MRC AIDS Reagent Repository. Recombinant MN gp120 (Genentech), SF-2 gp120 (Chiron) and CM243 gp120 were provided by the NIAID AIDS Reagents Repository, and W61D gp120 (SmithKline Beecham, Belgium) was from the UK MRC AIDS Reagent Repository.

Recombinant, monomeric JR-FL and LAI gp120s, both full-length and with the V3-loops deleted, were expressed using vectors developed at Progenics Pharmaceuticals that contain a dihydrofolate reductase expression cassette. The expression of the gene is under the control of the cytomegalovirus immediate-early promoter. For Δ-V3 gp120s, the V3 loops were excised by the splicing-by-overlap-extension technique, such that the cysteines defining the loop were retained and spanned by the peptide sequence TGAGH. All constructs were sequenced to verify that no mutations were introduced during the cloning manipulations. The proteins were expressed in stably transfected Chinese hamster ovary cells (DXB-11), selected in nucleoside-free medium and amplified using methotrexate, following previously described methods(17). The secreted proteins

were purified to >95% homogeneity in a non-denaturing process comprising an ion exchange, Galanthus nivalus lectin affinity and gel filtration chromatography. The purified proteins bound sCD4 with nanomolar affinity(18).

For expression of SF162 gp160, a 3.5-kb EcoR1-BamH1 fragment containing the env gene was excised from the SV40-based vector pSM and subcloned into the R1/Bg1111 sites of the β-actin-based expression vector pCAGGS. For expression of SF170 gp160, a 3.8-kb fragment containing the env gene was excised from the pBSKS^{+} plasmid, blunted by treatment with T4 DNA polymerase, and subcloned into the RV/Xho1 sites of pCAGGS. The expression plasmids were transfected into 293 T cells by calcium phosphate co-precipitation. Soluble gp120 in the culture supernatant was collected after three days, filtered through 0.2-μm filters and concentrated over an Amicon 1000 membrane.

The preparation of soluble, oligomeric forms of the JR-FL and 94RW020 envelope glycoproteins (and also monomeric gp120 from 92TH014) was as follows. The JR-FL env gene was provided by I. Chen (UCLA) and the env genes of 92TH014 and 92RW020 were obtained from the NIAID AIDS Reagent Repository(30). Soluble expression plasmids encoding gp120 and the gp41 ectodomain of JR-FL and 92RW020, gp120 only of 92TH014, were constructed as described(30), and transfected into Chinese hamster ovary cells by the calcium phosphate method. The cleavage sites between the JR-FL and 92TH014 gp120 and gp41 moieities were retained, and proteins secreted as oligomers (J.A., J.M.B. and J.P.M., unpublished data). Envelope glycoproteins were partially purified from culture supernatants by immobilized metal-affinity chromatography. A control preparation, 93MW959(c), containing a gp120/gp41 molecule incompetent at CD4 binding, by virtue of a single point mutation at residue 457, did not

compete with ^{125}I-MIP-1β. The monomeric gp120 or oligomeric gp120/gp41 concentrations in unpurified culture supernatants were estimated by denaturing the proteins(19), then dot-blotting onto nitrocellulose membranes and detecting the gp120 with a cocktail of murine monoclonal antibodies to continuous epitopes(19), followed by an anti-mouse IgG-HRP conjugate and the ECL chemiluminescence system (Amersham). Purified, monomeric JR-FL gp120 was used as a concentration standard(17). The concentration of oligomeric gp120/gp41 complexes was defined as the total concentration of monomeric gp120 subunits in the preparation. High-affinity CD4 binding of the gp120s was confirmed by enzyme-linked immunosorbent assay (ELISA)(19).

Cells and cell lines. PBMCs were isolated from blood donors by Ficoll-Hypaque centrifugation, and stimulated for 2-3 days with phytohaemagglutnin (5 μg ml^{-1}) and IL-2 (100 U ml^{-1}) (Roche). CD4$^+$ T cells were purified from the activated PBMCs by positive selection using anti-CD4 immunomagnetic beads (Dynal Inc.). The purified lymphocytes were cultured for at least 3 days at 2 x 10^6 / ml in medium containing IL-2 (200 U ml^{-1}) before being used in the ^{125}I-MIP-1β binding assay. The cells were screened for CCR-5-defective alleles(14), and only cells from wild-type donors were used (except when specified). 293 cells were transfected with pcDNA3.1-ckr-5 (ref. 1) using the calcium phosphate method, and resistant clones were selected in culture medium containing 1 μg ml^{-1} neomycin (G418; Sigma). Resistant cells were subcloned and tested for CCR-5 expression in a binding assay using ^{125}I-MIP-1β.

MIP-1β binding assay and gp120 competition. Purified CD4$^+$ T cells were washed twice in ice-cold binding buffer (RPMI 1640 medium containing 1% BSA, 25mM HEPES, 0.05% sodium azide). Duplicate samples (2 x 10^6 cells in 200 μl) were

incubated with 0.1 nM ^{125}I-MIP-1β (2,200 Ci mmol^{-1}; 0.25 μCml^{-1}) for 2 h on ice. Unlabelled ligand or gp120 (mixed with monoclonal antibodies when appropriate) was added to the cells immediately before radiolabelled ligand was added. Anti-CD4 monoclonal antibodies were added to the cells simultaneously with gp120. These cells were then separated from unbound ligand by centrifugation (60s, 14,000g) through oil (80% silicone oil, Aldrich; 20% mineral oil, Sigma), and the radioactivity in the cell pellet was determined by gamma counting. Specific binding of ^{125}I-MIP-1β was estimated by including a 100-fold excess of unlabelled MIP-1β. Each experiment was repeated at least twice using cells from different donors. For experiments with 293-CCR-5 cells, the cells were detached with 1mM EDTA then washed twice with binding buffer. Samples (5 x 10^5 cells) were incubated with 0.5 nM ^{125}I-MIP-1β, then processed as above. When the ^{125}I-MIP-1β concentration was reduced to 0.1 nM, no specific binding was detected.

Summary

The β-chemokine receptor CCR-5 is an essential co-factor for fusion of HIV-1 strains of the non-syncytium-inducing (NSI) phenotype with CD4$^+$ T-cells(1-5). The primary binding site for human immunodeficiency virus (HIV)-1 is the CD4 molecule, and the interaction is mediated by the viral surface glycoprotein gp120 (6, 7). The mechanism of CCR-5 function during HIV-1 entry has not been defined, but we have shown previously that its β-chemokine ligands prevent HIV-1 from fusing with the cell(1). We therefore investigated whether CCR-5 acts as a second binding site for HIV-1 simultaneously with or subsequent to the interaction between gp120 and CD4. We used a competition assay based on gp120 inhibition of the binding of the CCR-5 ligand, macrophage inflammatory protein (MIP)-1β, to its receptor on activated CD4$^+$ T cells or CCR-5 positive CD4$^-$ cells. We

conclude that CD4 binding, although not absolutely necessary for the gp120-CCR-5 interaction, greatly increases its efficiency. Neutralizing monoclonal antibodies against several sites on gp120, including the V3 loop and CD4-induced epitopes, inhibited the interaction of gp120 with CCR-5, without affecting gp120-CD4 binding. Interference with HIV-1 binding to one or both of its receptors (CD4 and CCR-5) may be an important mechanism of virus neutralization.

MIP-1β is the most specific ligand for CCR-5 (8-10) because MIP-1α and RANTES also bind with high affinity to other members of the β-chemokine receptor family on lymphoid cells(8-11). We therefore used MIP-1β as the CCR-5 ligand in the competition assays. In common with other members of this receptor family(12), CCR-5 is a mitogen-response gene. Its expression in quiescent, purified CD4$^+$ T-cells is usually minimal, but 3 days after activation of the cells by phytohaemagglutinin and interleukin (IL)-2, we observed strong increases in CCR-5 messenger RNA and ^{125}I-labelled-MIP-1β binding (data not shown). As specificity controls, we used CD4$^+$ T cells from individuals homozygous for defective CCR-5 alleles(13,14). The amount of specific (that is, cold MIP-1β-competed) ^{125}I-MIP-1β (0.1 nM) binding to cells from three such individuals was 92 $\pm$ 12 c.p.m. per 2 x 10^6 cells (mean $\pm$ s.d.). In contrast, mean binding to cells from 21 control individuals was 1,044 $\pm$ 1,073 c.p.m. per 2 x 10^6 cells (range, 222-4,846 c.p.m.). Most of the ^{125}I-MIP-1β reactivity with activated CD4$^+$ T cells therefore derives from binding to CCR-5.

When recombinant, monomeric gp120s were added with ^{125}I-MIP-1β to activated CD4$^+$ T cells, we found that gp120 from the NSI strain JR-FL [which used CCR-5 for entry(1)] strongly inhibited MIP-1β binding (Fig. 3a: Table 4).

TABLE 4 Effect of recombinant gp120 on MIP-1β binding

	gp120 ($\mu g\ ml^{-1}$)						
	0.1	0.2	.05	5	20	50	V3 sequence
NSI gp120							
JR-FL (B)	44±9	40±23	58±26	67±9	91±3	95±6	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC
JR-FL (B)*	46±28	56±3	84±11				
SF 162 (B)	81±8	113±41					CTRPNNNTRKSITIGPGRAFYATGDIIGDIRQAHC
W61D (B)	39±14	53±17	65±23		86±12		CTRPNNNTRKGIHIGPGRAFYAARKIIGDIRQAHC
92TH014 (B)	6±1	57±16	65±5				CTRPNNNTRKSIHLGPGRAWYTTGQIIGDIRQAHC
SF 170 (A)	87±45	140±49					CTRPNNNTRKSVRIGPGQAFYATGDIIGDIRQAYC
92RW020(A)*	21±39	49±22	74±36				CTRPNNNTRKGVRIGPGQAFYTGGIIGDIRQAHC
CM243 (E)	17±14	40±11	56±38		76±24		CTRPSNNTRPSITVGPGQVFYRTGDIIGDIRRAYC
TCLA gp120							
LAI (B)			-1±3	-5±36	-18±39	-6±9	CTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHC
MN (B)			-20±14	-23±4	1±0	0±6	CTRPNYNKKRIHIGPGRAFYTTKNIIGTIRQAHC
SF-2 (B)			-1±4	3±3	8±26	21±3	CTRPNNNTRKSIYIGPGRAFHTTGRIGDIRKAHC
gp41							
IIIB (B)				24±5			

Table 4 Legend:

Recombinant proteins were titrated in the presence of 0.1 nM ^{125}I-MIP-1β and added to activated CD4$^+$ T cells. Percentage inhibitions of ^{125}I-MIP-1β binding at each gp120 concentration are shown, and are the means $\pm$ s.d. of 2-4 independent experiments. No value indicates that the gp120 molecule was not tested at that concentration (several molecules were not available at concentrations > 1μg ml^{-1}).
* An oligomeric gp120/gp41 complex.

Half-maximal inhibition occurred in the range 0.1-1.0 $\mu g\ ml^{-1}$ (0.8-8nM) gp120, which is similar to the association constant for the gp120-CD4 interaction(7). In contrast, gp120 from the T-cell-line adapted (TCLA) SI strain LAI was ineffective (Fig. 3a; Table 4). This virus uses fusin (CXCR-4), but not CCR-5, for entry(1-5). Mutants of JR-FL and LAI gp120s which lack the V3 loop (Δ-V3 gp120), but bind CD4 with high affinity, did not block MIP-1β binding (Fig. 3a). However, peptides (15-residue if not specified) from the V3 loops of the following strains were also inactive: JR-FL (32-residue), RA, VS, Case-B (each NSI); HxB2, MN, SF-2 (each TCLA) (peptides were added at 1$\mu g\ ml^{-1}$, the approximate molar equivalent of 60 $\mu g\ ml^{-1}$ gp120). An oligomeric complex of JR-FL gp120 noncovalently associated with the ectodomain of gp41 was an effective inhibitor of MIP-1β binding, but a recombinant molecule comprising only the gp41 ectodomain was not (Table 4), although the latter molecule may not fold into a native structure(15).

HIV-1 strains from genetic subtypes A, B, C and E can use CCR-5 for entry(3), and we have found that MIP-1β inhibits the replication of most primary, NSI HIV-1 strains from subtypes A to E. This breadth of reactivity of HIV-1 with CCR-5 extend to the ^{125}I-MIP-1β competition assay. Recombinant gp120s from the following NSI primary strains were competitive, with half-maximal inhibition of MIP-1β binding occurring at concentrations around 0.1-0.5 $\mu g\ ml^{-1}$: JR-FL (subtype B), SF162 (B), W61D (B), 92TH014 (B), SF170 (A), 92RW020 (A) and CM243 (E) (Table 4). In contrast, no competition was observed with gp120s from the TCLA subtype B strains LAI, MN and SF-2 (Table 4), although each could bind CD4 with high affinity (not shown). Thus the phenotype of the virus from which a gp120 molecule is derived is more important than the viral genotype in determining interactions with CCR-5.

We assessed the role of CD4 in the competition between NSI gp120 and MIP-1β by using antagonists of the gp120-CD4 interaction. The monoclonal antibodies Q4120 and L77, which react with domain 1 of CD4 to inhibit gp120 binding(16), and soluble CD4 (sCD4), which reacts with gp120 to inhibit CD4 binding(17), both reversed the inhibition by JR-FL gp120 of MIP-1βbinding to CCR-5 (Fig. 3b; Table 5).

TABLE 5 Monoclonal antibody inhibition of the gp120 interaction with CCR-5

	Epitope	Monoclonal antibody	Inhibition (%)	±s.d.
CD4 antibodies	CD4-D1	Q4120	83	27
		L77	77	12
	CD4-D2	5A8	3	15
	CD4-D3	Q425	15	23
	CD4-D4	L120	-17	23
anti-gp120 antibodies non-neutralizing face	C5	D7324	-13	1
		23A	-11	14
	C1(D)	CRA-1	-2	18
		522-149	9	1
	C1(L)	133/192	8	7
	C1-C4	A32	51	11
		211c	5	11
	C1-C5	C11	3	21
anti-gp120 antibodies neutralizing face	CD4bs	sCD4*	91	18
	CD4bs	15e†	65	18
		IgG1b12*	125	40
	C4(L)	G3 508†	57	1
	C4-V3(D)	G3 42†	13	10
	CD4i	48d*	54	33
		17b*	79	41
	V2	697-D†	3	37
		SC258	-3	8
	V3	447-D*	109	2
		19b†	88	12
		83.1†	140	48
		2G12*	38	4

Table 5 Legend:

JR-FL gp120 (2ug ml^{-1}) inhibition of ^{125}I-MIP-1β binding to activated $CD4^+$ T cells was tested in the presence or absence of sCD4 (50 μg ml^{-1}) or monoclonal antibodies to CD4 (50 μg ml^{-1}) or antibodies to gp120(20μg ml^{-1}). Mean percentage reversals of the competitive effect of gp120 in the presence of each antibody (±s.d; n= 2-4 independent experiments) are shown. The level of specific ^{125}I-MIP-1β binding (c.p.m.) Recorded in the presence of gp120 but the absence of antibody was set at 0%, and the level recorded in the absence of both gp120 and antibody was set at 100%. A negative percentage reversal indicates that the competitive effect of gp120 on ^{125}I-MIP-1β binding was increased in the presence of the antibody. Also listed are the approximate positions of the antibody epitopes on gp120, as defined(19,20). References to the origin of the antibodies are described elsewhere(19,20) or listed in the Methods section.

* Anti-gp120 monoclonal antibodies (or sCD4) able to neutralize HIV-1_{JR-FL}.

† Monoclonal antibodies with neutralizing activity against other HIV-1 strains (primary or TCLA).

Monoclonal antibodies to other domains of CD4 [which do not block gp120-CD4 binding(16)] were ineffective (Table 5) and, in the absence of gp120, sCD4 (50 $\mu g\ ml^{-1}$) caused no inhibition of MIP-1β binding (data not shown). An interaction with cell-surface CD4 is therefore important for gp120 to interact efficiently with CCR-5 and block MIP-1β binding. To determine whether CD4 was an absolute requirement, we prepared a stable human $CD4^-$ $CCR\text{-}5^+$ 293 cell line. These cells bind ^{125}I-MIP-1β (specific binding up to 2,500 c.p.m. per 5 x 10^5 cells), whereas untransfected 293 cells do not (specific binding <50 c.p.m.). The binding of ^{125}I-MIP-1β to the $CD4^-$ $CCR\text{-}5^+$ 293 cells was sporadically inhibited by JR-FL gp120, but only at the highest gp120 concentrations tested (50-100 $\mu g\ ml^{-1}$). The strongest competition observed on these cells was 73% inhibition of MIP-1β binding by 50 $\mu g\ ml^{-1}$ of JR-FL gp120 (comparable inhibition was found in two other experiments), but competition was often not detected at all, and we never observed inhibition of MIP-1β binding at lower concentrations of gp120. The addition of excess sCD4 to the $CD4^-$ $CCR\text{-}5^+$ cells neither reduced nor increased the inhibitory effect of JR-FL gp120 (data not shown).

The interaction between JR-FL gp120 and CCR-5 requires at least 100-fold higher gp120 concentrations on $CD4^-$ cells than on $CD4^+$ cells. We suggest this is because binding of gp120 to CD4 on the cell surface increases the probability of a gp120-CCR-5 interaction; either a gp120-CD4-CCR-5 ternary complex forms, or there are sequential interactions of gp120 with CD4, then CCR-5. One possibility is that the high-affinity association of gp120 with CD4 increases the probability of a lower-affinity interaction of gp120 with CCR-5 (a proximity effect). This is consistent with the finding that sCD4 does not substitute for cell-surface CD4, at least with JR-FL gp120. Alternatively, binding to CD4

may be necessary to (better) expose a CCR-5 binding site on gp120. This may be especially important in the context of virions, where some regions of the oligomeric envelope glycoproteins (including the V3 loop) that are accessible on monomeric gp120 are not optimally exposed before CD4 binding(18).

To gain further sight into how the gp120-CD4 complex interacts with CCR-5 on activated $CD4^+$ T cells, we used a panel of HIV-1 neutralizing and non-neutralizing anit-gp120 monoclonal antibodies(19,20) having confirmed that each could bind to JR-FL gp120. The antibodies were tested for reversal of the competitive effect of gp120 on MIP-1β binding site (Table 5). As with sCD4, the antibodies to conformational (15e and IgG1b12) or linear (G3-508) epitopes overlapping the CD4-ginding site(20) prevented JR-FL gp120 from competing with MIP-1β. However, several antibodies that do not affect the binding of monomeric gp120 to CD4 (20) also inhibited the gp120-CCR-5 interaction (Table 5). These included three (447-D, 10b and 83.1) to the V3 loop; one (2G12) to a conserved epitope in the C3-V4 region; two (48d and 17b) to a conserved, CD4-induced epitope. All of these except A32 map to what we have defined as the gp120-neutralizing face(20). Eight other monoclonal antibodies that did not prevent JR-FL gp120 from blocking MIP-1β binding (Table 2) cluster on what we have defined as the gp120-non-neutralizing face(20): their epitopes are accessible on monomeric gp120, but in the context of the oligomer they are occluded either by other gp120 subunits or by gp41 molecules(19,20). These ineffective antibodies include 2/11c to an epitope overlapping that of A32; for this reason, and because A32 neutralizes no HIV-1 strains strongly, the significance of the partial inhibitory action of A32 on the gp120-CCR-5 interaction is uncertain. Two monoclonal antibodies (697-D and SC259) to the V2 loop were

also ineffective; although the V2 loop structure is modelled as being on (or above) the gp120 neutralizing face(20), these two antibodies are unable to neutralize HIV-1_{JR-FL}. The monoclonal antibodies 2G12, 17b, 447-D, 48d, IgG1b12, G3-508 and 697-D were also tested against the oligomeric JR-FL gp120/gp41 protein, and all except 697-D inhibited the interaction of this protein with CCR-5 (not shown).

Most of these antibodies to the neutralizing face of gp120 therefore either prevented gp120 from binding to CD4 or interfered with subsequent interactions with the CCR-5 second receptor. Not every antibody to this face of gp120 actually neutralizes HIV-1_{JR-FL}, as primary viruses resist neutralization, and studies with recombinant proteins can only predict neutralization efficiencies imprecisly(18). However, our findings may have implications for understanding how HIV-1 is neutralized by antibodies; blockade of the primary or secondary receptor interactions of the virus may be particularly important.

The simplest interpretation of the inability of Δ-V3 JR-FL gp120 to block MIP-1β binding (Fig. 3a) is that the CCR-5 binding site is contained within the V3 loop. This would be consistent with the many observations that the V3 loop contains important determinants of HIV-1 phenotype and tropism(18,21), and can influence second-receptor usage(3). We believe, however, that the CCR-5 binding site is not limited to the V3 loop. The V3 sequences of gp120s of subtypes A, B and E that interact with CCR-5 are rather variable (Table 4). Furthermore, some simian immunodeficiency virus (SIV) strains can also use human CCR-5 as a second receptor (Z.W. Chen and P. Marx, personal communication), but the V3 regions of HIV-1 and SIV have almost no primary sequence homology. Can all these sequences each form a binding site for the same, conserved

cellular protein, when similar V3 sequences from TCLA HIV-1 strains cannot (Table 4)? Twin-site models of the interaction of ligands with chemokine receptors(8) leave open the possibility that a relatively conserved section of the V3 loop could be one component of a multi-point binding site for CCR-5 on gp120. However, we suggest that the CCR-5 binding site must include a region of gp120 that is strongly conserved across the primate immunodeficiency viruses, not just across the HIV-1 genetic subtypes. Whether this is also the case for HIV-1 interactions with CXCR-4 remains to be determined.

The structure of the V3 loop may influence the nature of a complex binding site for CCR-5 on gp120. A region of gp120 overlapped by (but not necessarily identical to) the CD4-induced epitopes of monoclonal antibodies 48d and 17b is a good candidate for such a site. These antibodies recognize similar conformationally sensitive structures that are probably located around the bases of the V1, V2 and V3 loops(22,23). Deletion of the V3 loop from both HxBc2 gp120 and JR-FL gp120 destroys the 48d and 17b epitopes(22,23) (unpublished data), which may be relevant to the inability of the Δ-V3 JR-FL gp120 to interact with CCR-5 (Fig. 5A), and single amino-acid changes in the V3 and C4 regions of HIV-1_{LAI} also have major effects on the structure of these epitopes(24).

Further studies will be required to refine our understanding of the CCR-5 binding site. The efficiency with which β-chemokines inhibit the replication of NSI primary isolate in peripheral blood mononuclear cells (PMBCs) is dependent on strain but not subtype (unpublished data), suggesting, perhaps, that the degree of overlap between the gp120s and β-chemokine binding sites on CCR-5 varies between gp120s. If so, the CCR-5 binding site on gp120 might be more

flexible than the CD4 binding site. Finally, although sCD4 inhibited the interaction between JR-FL gp120 and CCR-5 on $CD4^+$ cells, for some strains of HIV-1 and (especially) HIV-2 and SIV, sCD4 might enhance the efficiency of second-receptor interactions, and thereby facilitate the entry of these primate immunodeficiency viruses into CD4 or $CD4^+$ cells(25,26).

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Third Series of Experiments

Summary

The CC-chemokine receptor CCR5 is required for the efficient fusion of M-tropic HIV-1 strains with the plasma membrane of CD4+ cells(1-5), and interacts directly with the viral surface glycoprotein gp120(6,7). Although receptor chimera studies have provided useful information(8-10), the domains of CCR5 that function for HIV-1 entry, including the site of gp120 interaction, have not been unambiguously identified. Here, we use site-directed, alanine-scanning mutagenesis of CCR5 to show that only substitutions of the negatively charged residues Asp-2 (D2), Asp-11 (D11) and Glu-18 (E18), singly or in combination, impair or abolish CCR5-mediated HIV-1 entry for the ADA and JR/FL M-tropic strains and the DH123 dual-tropic strain. These mutations also impair env-mediated membrane fusion and the gp120-CCR5 interaction. Of these three residues, only D11 is necessary for CC-chemokine-mediated inhibition of HIV-1 entry, which is, however, also dependent on other extracellular CCR5 residues. Thus, the gp120 and CC-chemokine binding sites on CCR5 are only partially overlapping, and the former requires negatively charged residues in the N-terminal CCR5 domain.

Result

To identify regions of CCR5 involved in gp120 binding and HIV-1 entry, we performed alanine-scanning mutagenesis of negatively (D, E) or positively (K, R, H) charged residues in the N-terminus (Nt) and three extracellular loops (ECL 1-3), on the grounds that charged residues have been previously implicated in the interactions of CC-chemokines with their receptors(11 12). We also altered any residues that differed between human CCR5 and its murine homologue (which is non-functional for HIV-1 entry)(9,10) whenever the difference involved a charge change. In all, 15 single, four double and one triple mutants were tested in these studies

(Fig.4).

The wt and mutant CCR5 proteins (HA-tagged at the C-terminus to facilitate detection) were transiently expressed in both U87MG-CD4 and SCL-1-CD4 cells and their abilities to support entry mediated by HIV-1 envelope glycoproteins were determined using an env-complementation assay with a luciferase readout (Fig. 5)(1,2). These non-lymphoid human cell lines were chosen because they lack the CCR5, CCR3 and CXCR4 co-receptors, so resist infection by HIV-1 in the absence of a transfected co-receptor(2,4,13,14) (a few exceptional HIV-1 strains will enter U87MG-CD4 cells via an unknown route(15), but we did not use them). Almost identical results were obtained with both cell lines. Two M-tropic viruses (ADA and JR-FL) that use CCR5 but not CXCR4(1-5), and one dual-tropic virus (DH123)(15) that uses both CCR5 and CXCR4 equally well(17), were used to test whether the mutant CCR5 proteins could support HIV-1 entry. The level of expression of each transfected CCR5 mutant was assessed by western blotting, and taken into account when determining co-receptor efficiency.

Of the 15 single mutations, only three had a significant inhibitory effect on the co-receptor function of CCR5 (Fig. 5). These were D2A, D11A and E18A, all located in the Nt domain of CCR5 (Fig. 4). The E18A substitution alone was sufficient to reduce CCR5 function by 15-20 fold. The double mutant D2A/D11A was less active than either of the single mutants, and the triple mutant (D2A/D1 1A/E18A) was almost completely inactive (>50-fold reduction in entry compared to wild-type) (Fig.5A). None of the other substitutions significantly affected CCR5 function in this assay (Fig.5B,C). Similar results were obtained with both M-tropic envelope glycoproteins, and the only difference noted with the dual-tropic DH123 envelope was a significantly increased

sensitivity to the D11A substitution (Fig.5A). Thus negatively charged residues in the CCR5 Nt have a major influence on the co-receptor function of this molecule.

To study the effects of the D2A, D11A and E18A substitutions in an independent assay of HIV-1 env function, we used a membrane fusion assay in which HeLa cells stably expressing the JR-FL env gene are mixed with HeLa-CD4 cells transiently transfected with wild-type or mutant CCR5 (Fig. 6)(1,18). The two cell types are labeled with different fluorescent probes, and fusion is monitored by resonance energy transfer (RET), which occurs only when the two dyes are present in the same membrane(1,18). We tested the D2A, D11A and E18A single mutants and the double and triple mutants, in comparison to wild type CCR5 in the RET assay. Each mutant had a phenotype in this fusion assay identical to what was observed in the viral entry assay (cf. Figs. 5A,6); the E18A and the double and triple mutants were completely unable to support env-mediated membrane fusion. However, when we boosted the expression of coreceptors by about 100-fold using the vaccinia-T7 polymerase (vFT7-pol) system(1,4.5,13), each of the CCR5 mutants was able to support membrane fusion, although less efficiently than the wild-type protein (Fig.6). We noted previously that CCR5 over-expression abolished the ability of its CC-chemokine ligands to inhibit membrane fusion, suggesting that some phenotypic changes can be missed if CCR5 expression is too high(1). The results with the vac-T7pol system do, however, show that even the triple mutant (D2A/D1 1A/E18A) is not completely inert as a co-receptor, just very strongly impaired.

We next tested whether the CCR5 mutants that supported HIV-1 entry were sensitive to the inhibitory effects of the CC-chemokine ligands of CCR5: MIP-1alpha, MIP-1β and RANTES (Table 1)(19-21)

Table 6

		MIP-1α	MIP-1β	RANTES
	Wt	100	100	100
Nt	D2A	81	97	85
	D11A	10	41	7
	N13A	121	93	92
	E18A	100	100	62
	K22A	19	12	-11
	K26A	100	97	100
	R31A	-5	2	-16
ECL1	Q93A	88	97	114
	D95A	107	112	121
	Q102A	107	98	93
ECL2	K171A/E172A	21	97	107
	H175A	119	100	95
	H181A	29	53	39
	Y184A	102	68	36
	Q188A	95	75	51
	K191A/N192A	14	15	18
ECL3	E262A	100	102	100
	R274A/D276A	48	36	33

Table 6: <u>Inhibition of co-receptor function by CC-chemokines</u> U87MG-CD4 cells were transiently lipofected with wild-type or mutant CCR5, then infected with NLluc/JR-FL, in the presence or absence of 2μg/ml of MIP-1α, MIP-1β or RANTES. Luciferase activity was measured 72h later(1.2). The relative percent inhibition by a CC-chemokine for each mutant is defined as [1-(luciferase c.p.s with chemokine/luciferase c.p.s. without chemokine)]/[1-(wild-type luciferase c.p.s with chemokine/wild-type luciferase c.p.s. without chemokine)] x

100%. Each value is a mean of 3 independent experiments, each performed in quadruplicate. Mutant co-receptors for which the relative percent inhibition is <50% of that observed with wild-type CCR5 are shaded.

(Note that although the D2A, D11A and E18A mutants, are impaired for HIV-1 entry, they did support enough entry for the sensitivity to inhibition to be determined. However, this was not true of the Nt double and triple mutants). In U87MG-CD4 cells, as with other non-lymphoid cells (1,2,22), the CC-chemokines do not completely block HIV-1 infection, and high concentrations are needed to obtain an effect. Thus we compared the degree of inhibition achieved by the CC-chemokines on the mutant and wild-type CCR5 receptors (40-60%, depending on the particular ligand, with individual potency being RANTES > MIP-1β > MIP-1α). The following mutants were relatively insensitive to the action of one or more of the CC-chemokines: D11A, K22A, R31A (Nt), H181A, Y184A, K171A/E172A, K191A/N192A (ECL2), R274A/D276A (ECL3). Of these, only D11A was impaired for both HIV-1 entry and CC-chemokine inhibition of entry. Amino acid substitutions at certain positions (mostly in the Nt and ECL2) do not, therefore, affect the HIV-1 co-receptor function of CCR5, but do affect CC-chemokine-mediated inhibition of this process (Table 6). The way in which these substitutions affect the action of the CC-chemokines has not yet been determined. However, the simplest interpretation is that the CC-chemokine binding site and the HIV-1 interactive site on CCR5 are not identical, and that certain substitutions in ECL2 and ECL3 affect only the CC-chemokine binding site.

To understand how the Nt substitutions affect the HIV-1 co-receptor function of CCR5, we determined whether they affected gp120 binding. We were unable to measure the binding of labeled gp120 to CCR5 directly, because the level

of CCR5 expression on transiently transfected cells was too low to obtain a reproducible signal in any of several binding assays tested. We therefore used a competition assay, in which the ability of gp120 (JR-FL)(7) to inhibit the binding of a phycoerythrin (PE)-labeled CCR5-specific MAb (2D7-PE)(23-25) was measured. The epitope for this MAb is located within ECL2, and we found that it was able to bind efficiently to HeLa cells co-transfected with CD4 and the CCR5 Nt mutants.

Independent studies show that 2D7 inhibits the binding of (125)I-labeled gp120 to CCR5 on the murine L1.2 cell line(25), which overexpresses CCR5 to an extent that permits the detection of gp120 binding(6,25). Here we show that the binding of 2D7-PE to wild type CCR5 was strongly inhibited (70%) by prior addition of gp120, indicating that the interaction of gp120 and 2D7 with the receptor is mutually exclusive (Fig. 7). However, gp120 only partially inhibited (40%) the binding of 2D7-PE to the D2A, D11A and E18A mutants, and was almost ineffective at blocking 2D7-PE binding to the double and triple Nt mutants (25% and 15% inhibition, respectively) (Fig. 7). Of note, those mutants most impaired for HIV-1 entry (Fig. 5) were also the ones for which 2D7-PE binding was least sensitive to gp120 inhibition (Fig. 7). The most probable explanation of this result is that gp120 binds to the wild type CCR5 molecule in such a way as to sterically hinder binding of 2D7-PE to ECL2, but binds poorly to the Nt mutants. A less likely possibility is that gp120 does bind efficiently to the Nt mutants but in an unusual orientation in which it is less able to inhibit 2D7-PE binding to ECL2. In the latter case, the geometry of inter-domain interactions in CCR5 has been altered by the Nt substitutions that impair CCR5 co-receptor function.

In this study, we have identified point substitutions at three negatively-charged residues in the amino-terminal domain that affect the co-receptor function of CCR5, without necessarily interfering with CC-chemokine inhibition of co-receptor function. The same substitutions affect the ability of gp120 to interact correctly with CCR5, probably by reducing the affinity of the gp120-CCR5 interaction. This may be sufficient to account for the co-receptor-defective phenotype. The loss of affinity for gp120 caused by the Nt substitutions in CCR5 can be partially compensated for by overexpressing the mutant co-receptors (Fig. 6), presumably because an increase in the number of low affinity co-receptors enables a successful gp120-CCR5 interaction to occur sufficiently rapidly to be compatible with the conformational changes in the envelope glycoproteins that initiate membrane fusion(26-28).

The gp120 binding site on CCR5 is therefore dependent on residues in the Nt, and it is possible that a discrete gp120-binding domain is actually confined to the Nt. Previous studies using chimeric receptors or deletion mutants indicated the importance of the CCR5 and CXCR4 Nt's for co-receptor function(8-10,29). The chimera studies also suggested that the site of interaction between CCR5 and HIV-1 is relatively broad and somewhat flexible(8-10). Although this possibility should not be discounted, alterations in the extracellular loops of receptor chimeras may also indirectly affect the orientation of the CCR5 Nt and hence its ability to interact correctly with gp120. In contrast to the gp120-binding site, the CC-chemokine binding site on CCR5 is dependent on residues in both the Nt and the extracellular loops (notably, but not exclusively, ECL2). Thus, although there is some overlap between the gp120 and CC-chemokine binding sites (as indicated by gp120 inhibition of CC-chemokine binding to CCR5)(6.7) they are not

identical, a conclusion consistent with studies showing that signal transduction and co-receptor activity are separable functions of CCR5(10,30,31).

A more detailed understanding of the interactive sites on CCR5 for gp120 and the CC-chemokines (and on these molecules for CCR5) will be required to define how HIV-1 uses CCR5 for entry into its target cells and, perhaps, for the development of inhibitors of this process. It will also be important to determine whether the negatively charged residues that we have identified in the CCR5 Nt interact directly or indirectly with positively charged amino acids in gp120, in the V3 loop and/or elsewhere.

METHODS

Lipofections and reporter gene assays Mutated cDNAs were subcloned into the pcDNA3.1 (Stratagene) expression vector. U87MG-CD4 and SCL-1-CD4 cells were incubated with lipofectin (5μg/ml) and pSVlacZ (5μg/ml), or mutant DNA (4μg/ml) + pSVlacZ (1μg/ml) in OPTI-MEM (Gibco BRL), for 5h at 37°C. The cells were infected 24h later with NLluc/Env supernatants, containing 200-500 ng/ml p24, for 2h at 37°C. For CC-chemokine blocking of HIV-1 entry, 2μg/ml of MIP-1α, MIP-1β or RANTES (R & D Systems) was added simultaneously with HIV-1 (50-100ng/ml p24), and maintained in the cultures for 12h. Cell samples were treated with 100μl of lysis buffer (Promega) 72h after infection, and luciferase (luc) and β-galactosidase activity (OD_{420}) were measured(1,2). Standardized luciferase activity is defined as (luc c.p.s / ng/ml p24 / (OD_{420} ÷ control OD_{420}) / (r.f.e. for mutant CCR5 bands ÷ r.f.e. for wild-type CCR5 bands) (see below). Luc c.p.s. values ranged from $5x10^5$ to $2x10^6$ for wild-type CCR5.

Immunoblot analysis of CCR5 expression in whole cell extracts All CCR5 molecules used in this study had a 9-residue hemagglutinin (HA)-tag as a C-terminal extension, to facilitate detection. Lipofected U87MG-CD4 cells from a 60mm tissue culture plate were resuspended in 1% sodium dodecyl maltoside, 10mM Tris-HCI (pH 6.8), 50mM NaCl, 1mM $CaCl_2$, 0.1mM PMSF, 5μg/ml leupeptin, 10μg/ml aprotinin, 0.7μg/ml pepstatin and 10mM EDTA. The suspension was incubated at 4°C for 30 min and the supernatant fraction collected. Total protein concentration was determined using the Bio-Rad DC Protein Assay. Protein (15μg) was then fractionated, without prior boiling, on an SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P membranes (Millipore) and probed for CCR5 with rabbit anti-HA-tag antibody (1:500 dilution; Berkeley Antibody Company) and AP-labeled goat anti-rabbit IgG (1:10^4 dilution; Amersham), followed by incubation with chemifluorescent substrate (Vistra ECF, Amersham). Relative fluorescence emission (r.f.e.) of immunoreactive bands, excited at 450nm, was detected on a laser-based scanner (Molecular Dynamics Storm 860). Identical expression patterns were obtained with whole-cell extracts and plasma-membrane extracts. CCR5 mutant expression levels varied from 20% to 100% of the wild-type protein. The relationship between wild-type CCR5 expression levels and HIV-1 entry efficiency was determined to be linear over a 10-fold range (data not shown).

Competition between gp120 and 2D7 MAb for CCR5 binding HeLa cells ($2x10^6$) were incubated for 5h with lipofectin (10μg/ml) and the pCDM8 CD4 expression vector (3.75μg/ml) plus wild-type or mutant CCR5 plasmids (1.25μg/ml) in OPTI-MEM. The cells were then infected for 12h with $2x10^7$ p.f.u. of vFT7 to boost CCR5 expression(1), detached with 2mM EDTA/PBS, and washed once with binding buffer (1% BSA,

0.05% azide in DPBS). Cells ($1x10^6$) were incubated for 1h at 37°C with or without 10μg/ml gp120 (JR-FL)(7) before addition of PE-labeled 2D7 MAb(23,24) (20ng/ml) for 30 min at 4°C. The cells were washed once with binding buffer and once with PBS, resuspended in 1% formaldehyde/PBS and analyzed by FACS to determine mean fluorescence intensity (m.f.i.). CD4 expression was monitored by staining with Leu3A, and varied by no more than ± 10% between samples. CCR5 mutant expression levels ranged from 20% to 100% of that of wild type CCR5.

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Fourth Series of Experiments

Direct Binding of HIV-1_{JR-FL} gp120 to CCR5$^+$ CD4$^-$ Cells

The direct binding of HIV-1_{JR-FL} gp120 to CCR5$^+$ CD4$^-$ cells has been demonstrated. In this case, preincubation of the gp120 with sCD4 or another CD4-based molecule is required, presumably because this results in a conformational change in gp120 that exposes a chemokine receptor binding site. Figure 8 illustrates the use of flow cytometry to measure the direct binding of sCD4/gp120 complexes to human CCR5-bearing murine L1.2 cells. Background levels of binding were observed with either biotinylated protein alone, or if gp120 from the laboratory-adapted strain HIV-1_{LAI} is used in place of the HIV-1_{JR-FL} gp120 (data not shown).

This assay has been adapted for drug screening purposes to a 96-well microplate format where binding of the sCD4/gp120 complexes to CCR5$^+$/CD4$^-$ cells is measured using a fluorometric plate reader. One method is as follows:

1) Plate out L1.2-CCR5$^+$ cells (approx. 500,000/well).
2) Add inhibitor for 1 hour at room temperature.
3) Wash and add biotinylated sCD4 (2.5μg/ml) and biotinylated HIV-1_{JR-FL} gp120 (5μg/ml), then incubate for 2 hours at room temperature.
4) Wash and incubate with streptavidin-phycoerythrin (100ng/nl).
5) Wash and measure the amount of bound gp120/sCD4 using a fluorometric plate reader exciting at 530nm and reading emission at 590nm.

Using this method, inhibition of binding of gp120/sCD4 to CCR5 by CC-chemokines (Fig. 9) and antibodies to CCR5 that block HIV-1 infection (not shown) have been demonstrated.

Inhibition of HIV-1 envelope-mediated membrane fusion by extracellular domains of CCR5.

Synthetic peptides representing the four extracellular domains of human CCR5 were made by Quality Controlled Biochemicals (Hopkinton, MA) and tested for ability to inhibit membrane fusion mediated by the envelope glycoproteins of the LAI or JR-FL strains of HIV-1 using the resonance energy transfer (RET) assay described above. Specific inhibition of fusion mediated by the JR-FL envelope glycoprotein was seen using the ECL2 peptide but not other peptides. ECL2 inhibited fusion between HeLa-env_{JR-FL} cells and PM1 cells by 97% at 100 μg/ml, 65% at 33 μg/ml and 15% at 11 μg/ml (mean of two assays). ECL2 gave no inhibition of fusion between HeLa-env_{LAI} and PMI cells or HeLa-env_{LAI} and HeLa-CD4 cells. These results strongly suggest that CCR5 ECL2 specifically inhibits fusion, most likely by blocking the interaction between HIV-1_{JR-FL} gp120 and CCR5. No other peptides tested gave significant levels of specific inhibition of fusion.

Inhibition of HIV-1 envelope-mediated membrane fusion by the bicyclam, JM3100.

The bicyclam JM3100, obtained from Dr. J. Moore (Aaron Diamond AIDS Research Center, NY) was tested for ability to inhibit membrane fusion mediated by the envelope glycoproteins of the LAI or JR-FL strains of HIV-1 using the resonance energy transfer (RET) assay described above. As illustrated in Fig. 10, this molecule specifically and potently inhibits fusion mediated by gp120/gp41 from the HIV-1_{LAI} strain, and not from the HIV-1 $_{JR-FL}$ strain. These data suggest that this molecule specifically inhibits HIV fusion by blocking the interaction between HIV-1_{LAI} gp120 and CXCR4.

SEQUENCE LISTING

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(2) INFORMATION FOR SEQ ID NO:1:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAGGCTACT TCCCTGATTG GCAGAACTAC ACACCAGG 38

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCAAGCCGA GTCCTGCGTC GAGAG 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGACTTTCC GCTGGGGACT TTC 23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCTGTTCGGG CGCCACTGCT AGAGATTTTC CAC 33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Asp Tyr Gln Val Ser Ser Pro Ile Tyr Asp Ile Asn Tyr Tyr Thr
1               5                   10                  15

Ser Glu Pro Cys Gln Lys Ile Asn Val Lys Gln Ile Ala Ala Arg
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

His Tyr Ala Ala Ala Gln Trp Asp Phe Gly Asn Thr Met Cys Gln
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Arg Ser Gln Lys Glu Gly Leu His Tyr Thr Cys Ser Ser His Phe Pro
1 5 10 15

Tyr Ser Gln Tyr Gln Phe Trp Lys Asn Phe Gln Thr Leu Lys Ile Val
20 25 30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gln Glu Phe Phe Gly Leu Asn Asn Cys Ser Ser Ser Asn Arg Leu Asp
1 5 10 15

Gln

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAGCTTGGAG AACCAGCGGT TACCATGGAG GGGATC 36

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTCTGAGTCT GAGTCAAGCT TGGAGAACCA 30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTCGAGCATC TGTGTTAGCT GGAGTGAAAA CTTGAAGACT C 41

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTCTGAGTCT GAGTCCTCGA GCATCTGTGT 30

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAGCTTCAGA GAGAAGCCGG GATGGAAACT CC 32

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTCTGAGTCT GAGTCAAGCT TCAGAGAGAA 30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTCGAGCTGA GTCAGAACCC AGCAGAGAGT TC 32

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTCTGAGTCT GAGTCCTCGA GCTGAGTCAG 30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AAGCTTCAGT ACATCCACAA CATGCTGTCC AC 32

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCTGAGTCT GAGTCAAGCT TCAGTACATC 30

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTCGAGCCTC GTTTTATAAA CCAGCCGAGA C 31

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTCTGAGTCT GAGTCCTCGA GCCTCGTTTT 30

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AAGCTTCAGG GAGAAGTGAA ATGACAACC 29

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GTCTGAGTCT GAGTCAAGCT TCAGGGAGAA 30

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCGAGCAGA CCTAAAACAC AATAGAGAGT TCC 33

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTCTGAGTCT GAGTCCTCGA GCAGACCTAA 30

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AAGCTTCTGT AGAGTTAAAA AATGAACCCC ACGG 34

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GTCTGAGTCT GAGTCAAGCT TCTGTAGAGT 30

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTCGAGCCAT TTCATTTTTC TACAGGACAG CATC 34

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTCTGAGTCT GAGTCCTCGA GCCATTTCAT 30

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GTCTGAGTCT GAGTCAAGCT TAACAAGATG GATTATCAA 39

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTCTGAGTCT GAGTCCTCGA GTCCGTGTCG CAAGCCCAC 39

What is claimed is:

1. A polypeptide having a sequence corresponding to the sequence of a portion of a chemokine receptor and capable of inhibiting the fusion of HIV-1 to $CD4^+$ cells and thus of inhibiting HIV-1 infection of the cells.

2. A polypeptide having a sequence corresponding to the sequence of a portion of the chemokine receptor, CCR5 and capable of inhibiting the fusion of HIV-1 to $CD4^+$ cells and thus of inhibiting HIV-1 infection of the cells.

3. The polypeptide of claim 2 comprising amino acids having a sequence of at least one extracellular domain of CCR5.

4. The polypeptide of claim 3 wherein the extracellular domain is the second extracellular loop.

5. A pharmaceutical composition comprising an amount of the polypeptide of claim 1 effective to inhibit the fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

6. A polypeptide having a sequence corresponding to that of a portion of a HIV-1 envelope glycoprotein capable of specifically binding to the chemokine receptor CCR5.

7. The polypeptide of claim 6, wherein the glycoprotein is gp120.

8. A pharmaceutical composition comprising an effective amount of the polypeptide of claim 6 effective to inhibit the fusion of HIV-1 to $CD4^+$ cells and a

pharmaceutically acceptable carrier.

9. An antibody or a portion of an antibody capable of binding to a chemokine receptor on a $CD4^{+}$ cell and inhibiting HIV-1 infection of the cell.

10. A pharmaceutical composition comprising an amount of the antibody of claim 9 effective to inhibit HIV-1 infection of $CD4^{+}$ cells and a pharmaceutically acceptable carrier.

11. A method of treating an HIV-1 infected subject which comprises administering to the subject the polypeptide of any of claims 1, 2, 3, 4, 6, or 7 in an amount effective to inhibit the fusion of HIV-1 to $CD4^{+}$ cells of the subject and thus treat the subject.

12. A method of reducing the likelihood of a subject from becoming infected by HIV-1 which comprises administering to the subject the polypeptide of any of claims 1, 2, 3, 4, 6, or 7 in an amount effective to inhibit the fusion of HIV-1 to CD4+ cells of the subject and thus reduce the likelihood of HIV-1 infection.

13. A method for inhibiting HIV-1 infection of $CD4^{+}$ cells which comprises contacting such $CD4^{+}$ cells with a non-chemokine agent capable of binding to the chemokine receptor CCR5 in an amount and under conditions such that fusion of HIV-1 to the $CD4^{+}$ cells is inhibited, thereby inhibiting HIV-1 infection of the cells.

14. The method of claim 13, wherein the non-chemokine agent is an oligopeptide.

15. The method of claim 13, wherein the non-chemokine agent is a polypeptide.

16. The method of claim 13, wherein the non-chemokine agent is a nonpeptidyl agent.

17. A non-chemokine agent capable of binding to the chemokine receptor CCR5 and inhibiting the fusion of HIV-1 to $CD4^+$ cells.

18. A pharmaceutical composition comprising an amount of the non-chemokine agent capable of binding to the chemokine receptor CCR5 and inhibiting the fusion of HIV-1 to $CD4^+$ cells effective to inhibit HIV-1 infection of $CD4^+$ cells and a pharmaceutically acceptable carrier.

19. A molecule capable of binding to the chemokine receptor CCR5 and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a ligand capable of binding to a cell surface receptor of the $CD4^+$ cells other than the chemokine receptor such that the binding of the non-chemokine agent to the chemokine receptor does not prevent the binding of the ligand to the other receptor.

20. The molecule of claim 18, wherein the cell surface receptor is CD4.

21. The molecule of claim 18, wherein the ligand comprises an antibody or a portion of an antibody.

22. A molecule capable of binding to the chemokine receptor CCR5 and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising a non-chemokine agent linked to a compound

capable of increasing the *in vivo* half-life of the non-chemokine agent.

23. The molecule of claim 21, wherein the compound is polyethylene glycol.

24. A pharmaceutical composition comprising an amount of the molecule of claim 19, 20, 21, 22 or 23 effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

25. A method for reducing the likelihood of HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 19, 20, 21, 22 or 23 to the subject.

26. A method for treating HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 19, 20, 21, 22 or 23 to the subject.

27. A method for determining whether a non-chemokine agent is capable of inhibiting the fusion of HIV-1 to a $CD4^+$, $CCR5^+$ cell which comprises:

(a) contacting the $CD4^+$, $CCR5^+$ cell, after it is labeled with a first dye, with a cell expressing an appropriate HIV-1 envelope glycoprotein on its surface, and labeled with a second dye, in the presence of an excess of the agent under conditions permitting fusion of the $CD4^+$, $CCR5^+$ cell to the cell expressing the HIV-1 envelope glycoprotein on its surface in the absence of an agent known to inhibit fusion of HIV-1 to $CD4^+$, $CCR5^+$ cells, the first and second dyes being selected so as to allow resonance energy transfer between the dyes;

(b) exposing the product of step (a) to conditions which would result in resonance energy transfer if fusion has occurred; and

(c) determining whether there is resonance energy transfer, the absence or reduction of transfer indicating that the agent is capable of inhibiting fusion of HIV-1 to $CD4^+$ and $CCR5^+$ cells.

28. The method of claim 27, wherein the agent is an oligopeptide, a polypeptide or a nonpeptidyl agent.

29. The method of claim 27, wherein the $CD4^+$ cell is a PM1 cell.

30. The method of claim 27, wherein the cell expressing the HIV-1 envelope glycoprotein is a HeLa cell expressing HIV-1_{JR-FL} gp120/gp41.

31. A transgenic nonhuman animal which comprises an isolated DNA molecule encoding the chemokine receptor CCR5.

32. The transgenic nonhuman animal of claim 31 further comprising an isolated DNA molecule encoding a sufficient portion of the CD4 molecule to permit binding the HIV-1 envelope glycoprotein.

33. A transgenic nonhuman animal which comprises an isolated DNA molecule encoding the chemokine receptor CCR5 and an isolated DNA molecule encoding fusin.

34. The transgenic nonhuman animal of claim 33 further comprising an isolated DNA molecule encoding a sufficient portion of the CD4 molecule to permit

binding the HIV-1 envelope glycoprotein.

35. A transformed cell which comprises an isolated nucleic acid molecule encoding the chemokine receptor CCR5.

36. An agent capable of inhibiting HIV-1 infection and capable of binding to a chemokine receptor without substantially affecting the said chemokine receptor's capability to bind to chemokines.

37. The agent of claim 36, wherein the said chemokine receptor is CCR5.

38. The agent of claim 36, wherein after the binding of the agent to the said chemokine receptor, a two fold higher concentration of the chemokine is required to achieve the degree of binding observed if the chemokine receptor had not been bound to the agent.

39. The agent of claim 36, wherein after the binding of the agent to the said chemokine receptor, a ten fold higher concentration of chemokine is required to achieve the degree of binding observed if the chemokine receptor had not been bound to the agent.

40. The agent of claim 36, wherein the agent is an oligopeptide, a nonpeptidyl agent or a polypeptide.

41. The agent of claim 40, wherein the polypeptide is an antibody or a portion of an antibody.

42. A pharmaceutical composition comprising an amount of the agent of claim 37, 38, 39, 40 or 41 effective to inhibit fusion of HIV-1 infection and a pharmaceutically acceptable carrier.

43. A method for inhibiting HIV-1 infection of $CD4^+$ cells which comprises contacting such $CD4^+$ cells with an agent capable of inhibiting HIV-1 infection and capable of binding to a chemokine receptor without substantially affecting the said chemokine receptor's capability to bind to chemokines.

44. A molecule capable of binding to the chemokine receptor CCR5 and inhibiting fusion of HIV-1 to $CD4^+$ cells comprising the agent of claim 36 linked to a compound capable of increasing the *in vivo* half-life of the non-chemokine agent.

45. The molecule of claim 44, wherein the compound is polyethylene glycol.

46. A pharmaceutical composition comprising an amount of the molecule of claim 44 or 45 effective to inhibit fusion of HIV-1 to $CD4^+$ cells and a pharmaceutically acceptable carrier.

47. A method for reducing the likelihood of HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 42 or 46 to the subject.

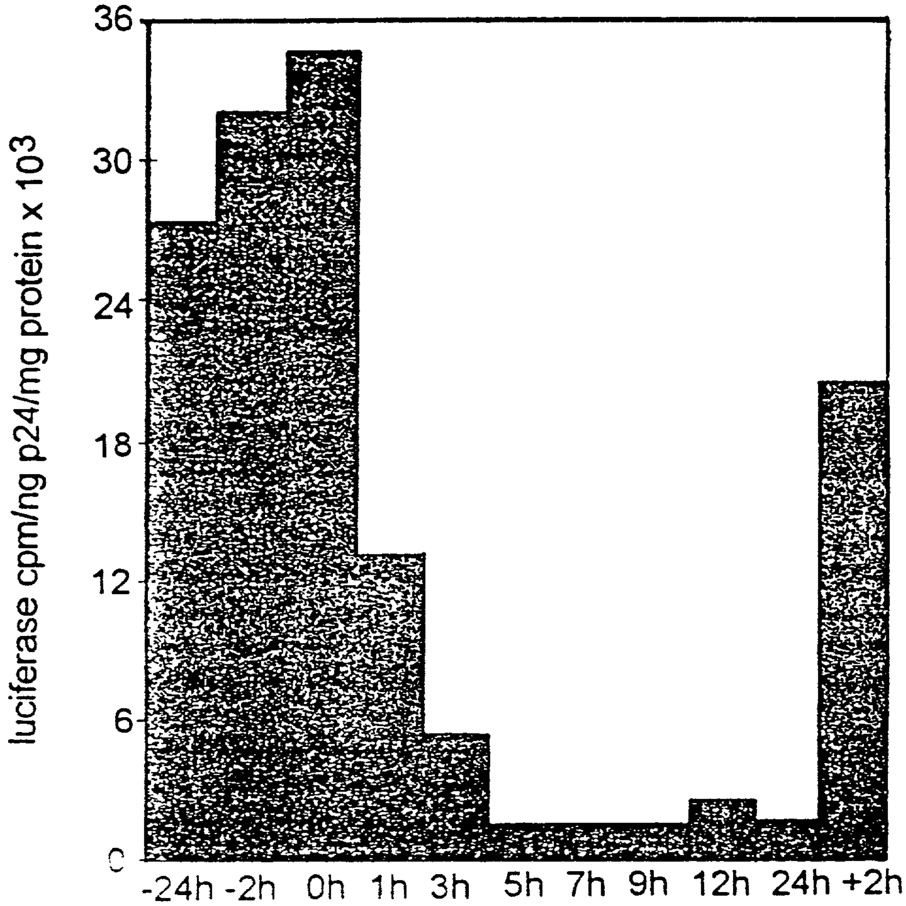
48. A method for treating HIV-1 infection in a subject comprising administering the pharmaceutical composition of claim 42 or 46 to the subject.

USES OF A CHEMOKINE RECEPTOR
FOR INHIBITING HIV-1 INFECTION

Abstract of the Disclosure

This invention provides a polypeptide comprising a fragment of a chemokine receptor capable of inhibiting HIV-1 infection. In an embodiment, the chemokine receptor is C-C CKR-5. In another embodiment, the fragment comprises at least one extracellular domain of the chemokine receptor C-C CKR-5. This invention further provides different uses of the chemokine receptor for inhibiting HIV-1 infection.

FIG. 1A



36
30
24
18
12
6
0
luciferase cpm/ng p24/mg protein x 103
-24h -2h 0h 1h 3h 5h 7h 9h 12h 24h +2h

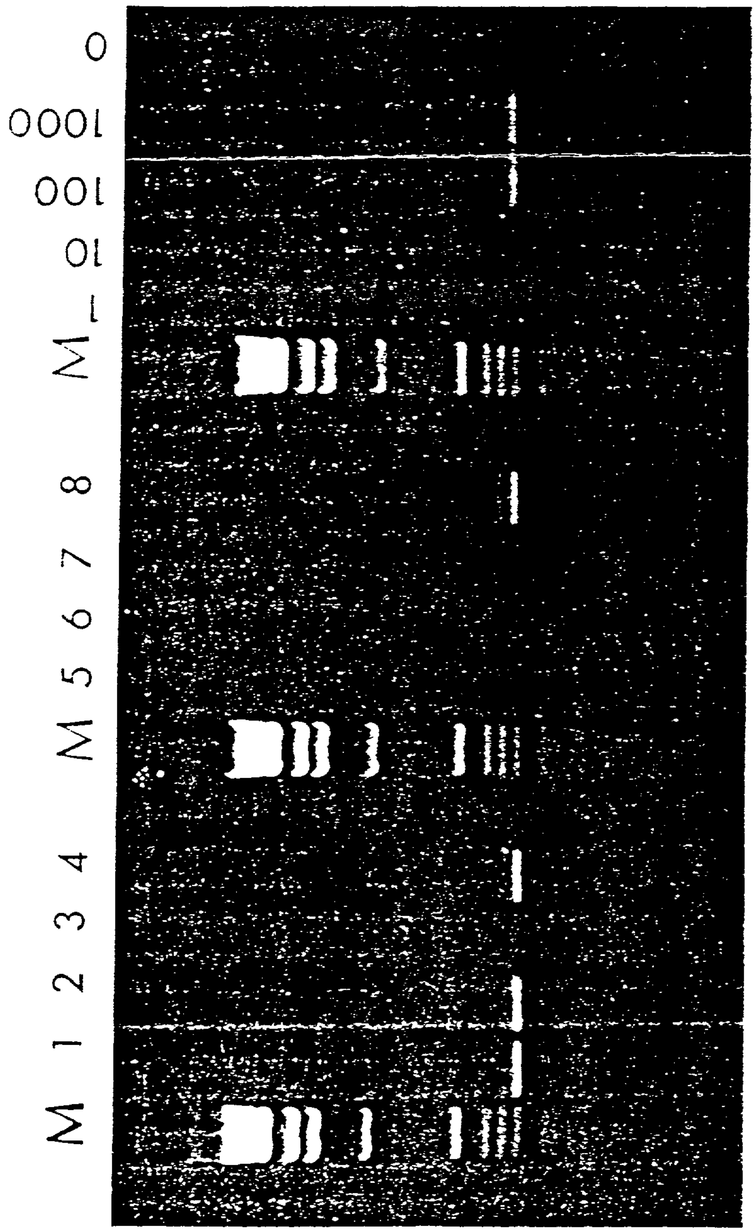


FIG. 1B

FIG. 2

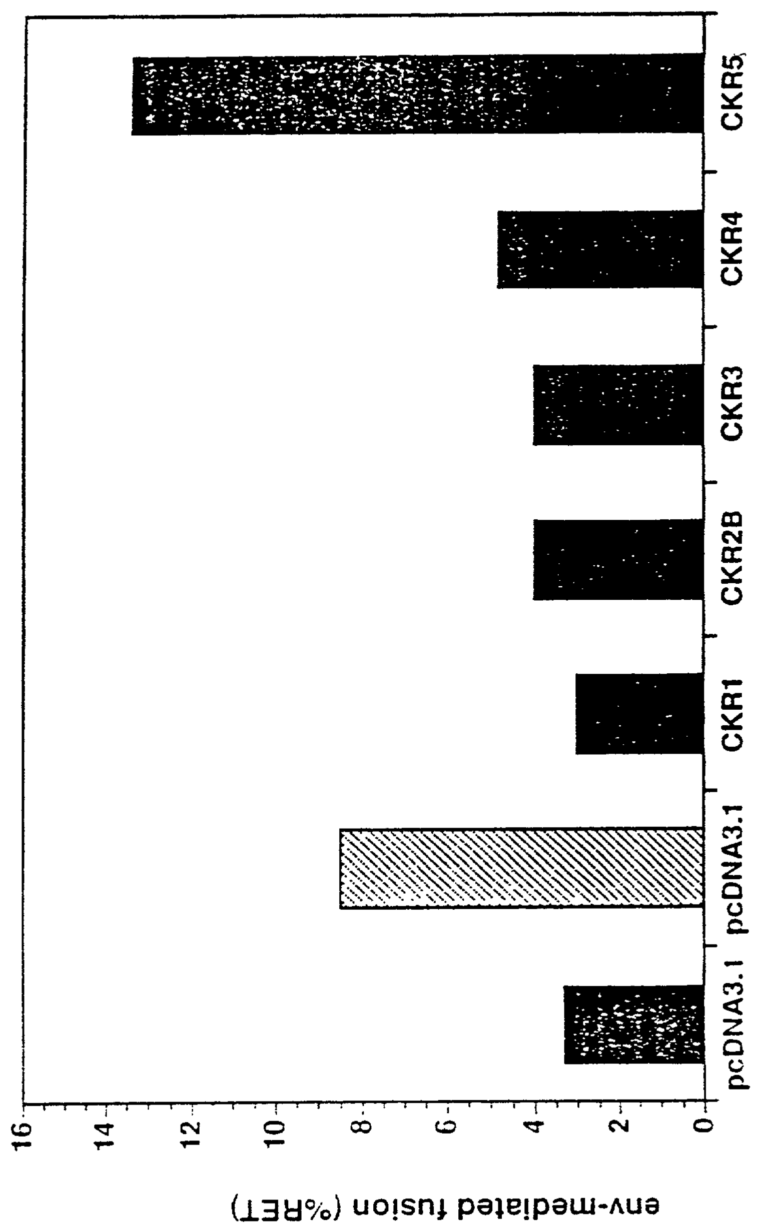

env-mediated fusion (%RET)
0
2
4
6
8
10
12
14
16
pcDNA3.1
pcDNA3.1
CKR1
CKR2B
CKR3
CKR4
CKR5

FIG. 3A

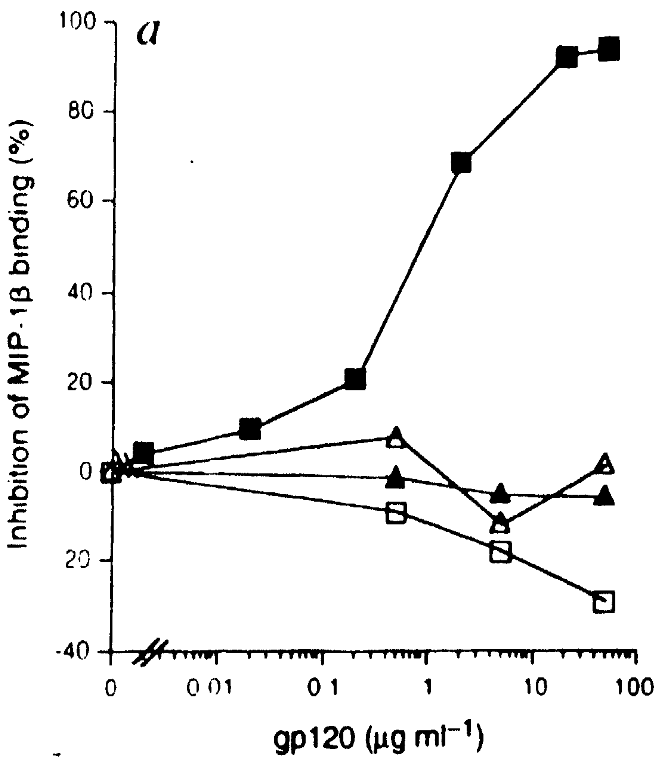

a
Inhibition of MIP-1β binding (%)
100
80
60
40
20
0
20
-40
0
0.01
0.1
1
10
100
gp120 (μg ml⁻¹)

FIG. 3B

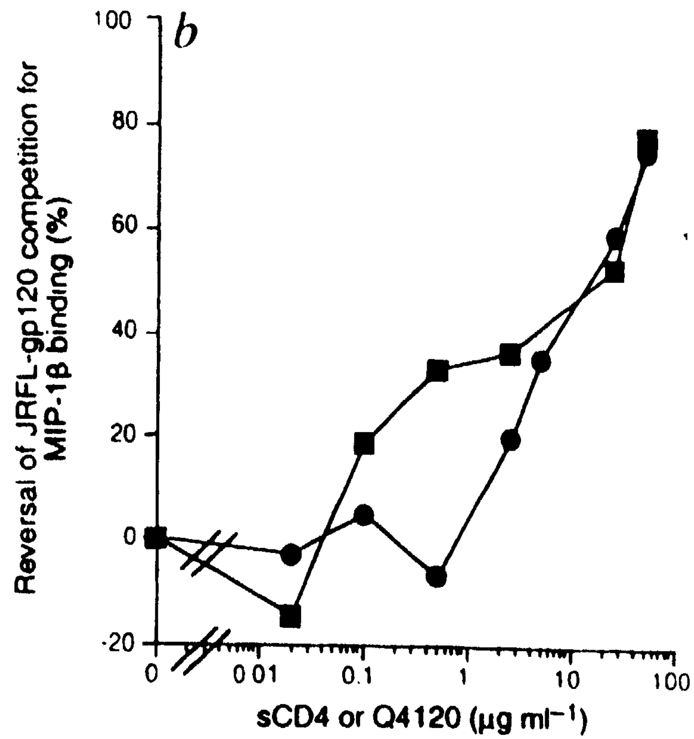

b
Reversal of JRFL-gp120 competition for MIP-1β binding (%)
100
80
60
40
20
0
-20
0
0.01
0.1
1
10
100
sCD4 or Q4120 (μg ml⁻¹)

FIG. 4

Nt:

	1									10										20										30	
Nt:	M	D	Y	Q	V	S	S	P	I	Y	D	I	N	Y	Y	T	S	E	P	C	Q	K	I	N	V	K	Q	I	A	A	R
		-	F		G		V		T		-		D		G	M		A/-				+				+					Q/+

ECL1:

			90										100		
ECL1:	H	Y	A	A	A	Q	W	D	F	G	N	T	M	C	Q
					N	E		V/-				I			K

ECL2:

			170										180										190				
ECL2:	R	S	Q	K	E	G	L	H	Y	T	C	S	S	H	F	P	Y	S	Q	Y	Q	F	W	K	N	F	Q
				+	-		F	+					P	+			H	T			H			+	S		

ECL3:

	261									270							
ECL3:	Q	E	F	F	G	L	N	N	C	S	S	S	N	R	L	D	Q
		-												+		-	

FIG. 5A

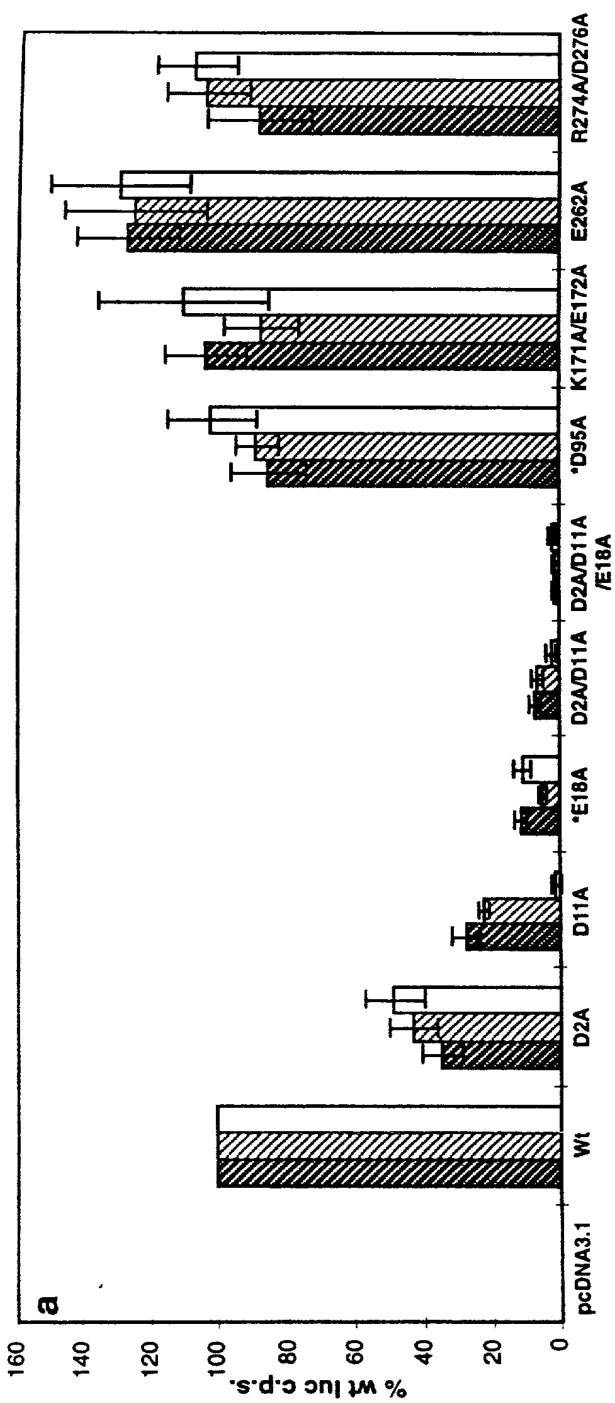

a
% wt luc c.p.s.
0
20
40
60
80
100
120
140
160
pcDNA3.1
Wt
D2A
D11A
*E18A
D2A/D11A
D2A/D11A /E18A
*D95A
K171A/E172A
E262A
R274A/D276A

FIG. 5B

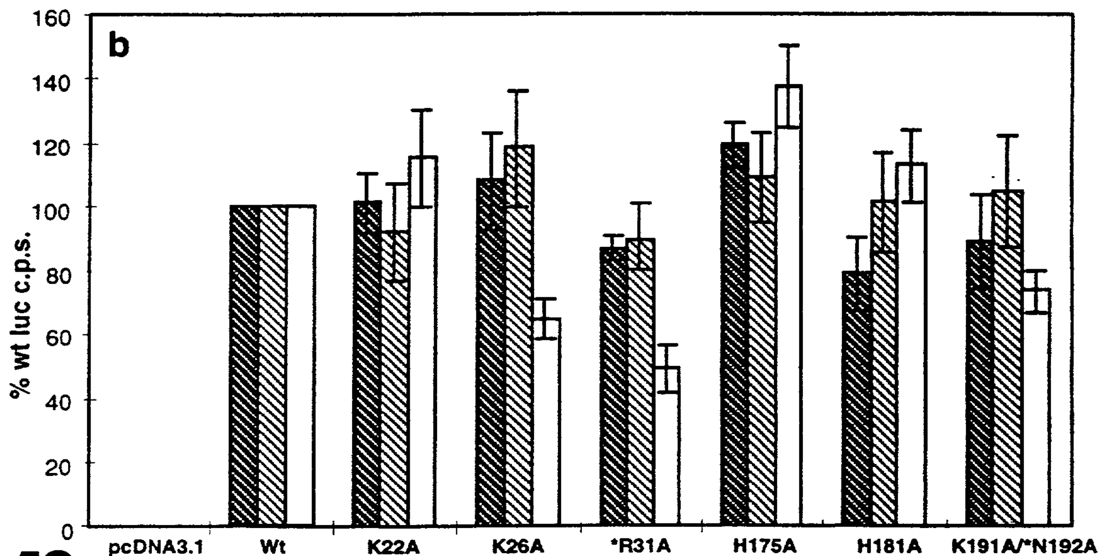

b
160
140
120
100
80
60
40
20
0
% wt luc c.p.s.
pcDNA3.1
Wt
K22A
K26A
*R31A
H175A
H181A
K191A/*N192A

FIG. 5C

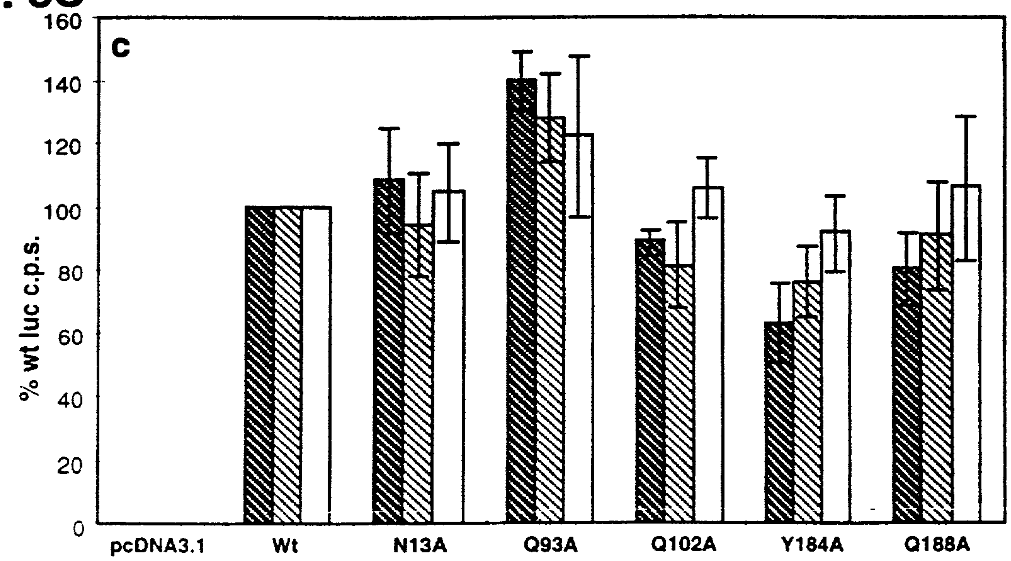

c
160
140
120
100
80
60
40
20
0
% wt luc c.p.s.
pcDNA3.1
Wt
N13A
Q93A
Q102A
Y184A
Q188A

FIG. 6

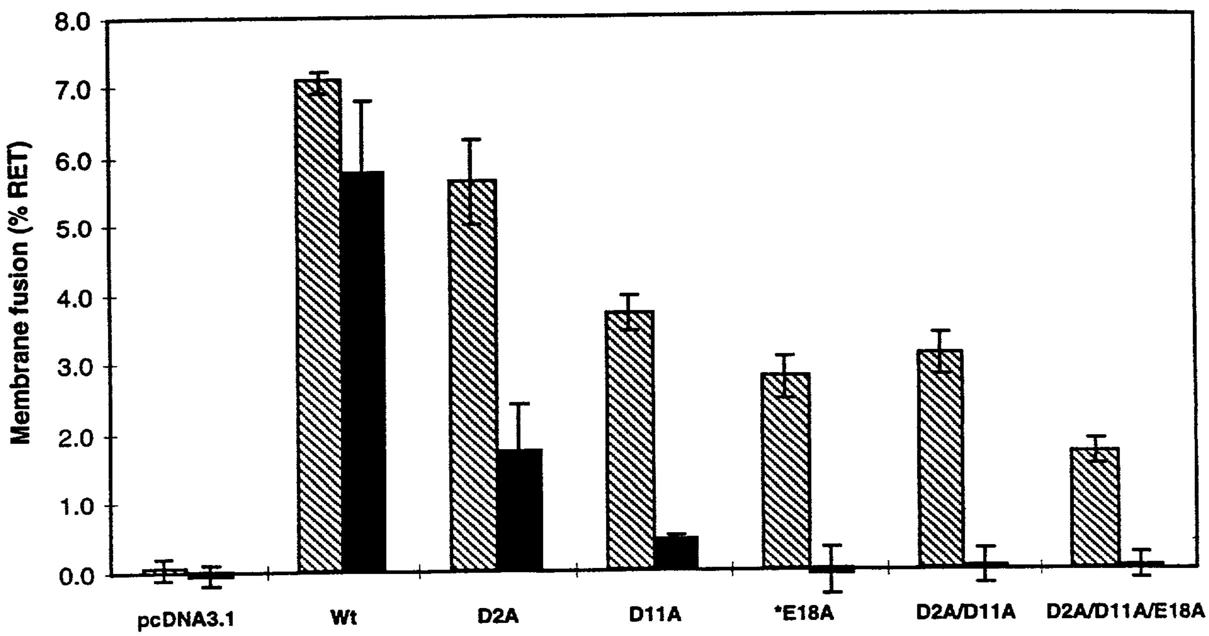

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Membrane fusion (% RET)
pcDNA3.1
Wt
D2A
D11A
*E18A
D2A/D11A
D2A/D11A/E18A

FIG. 7

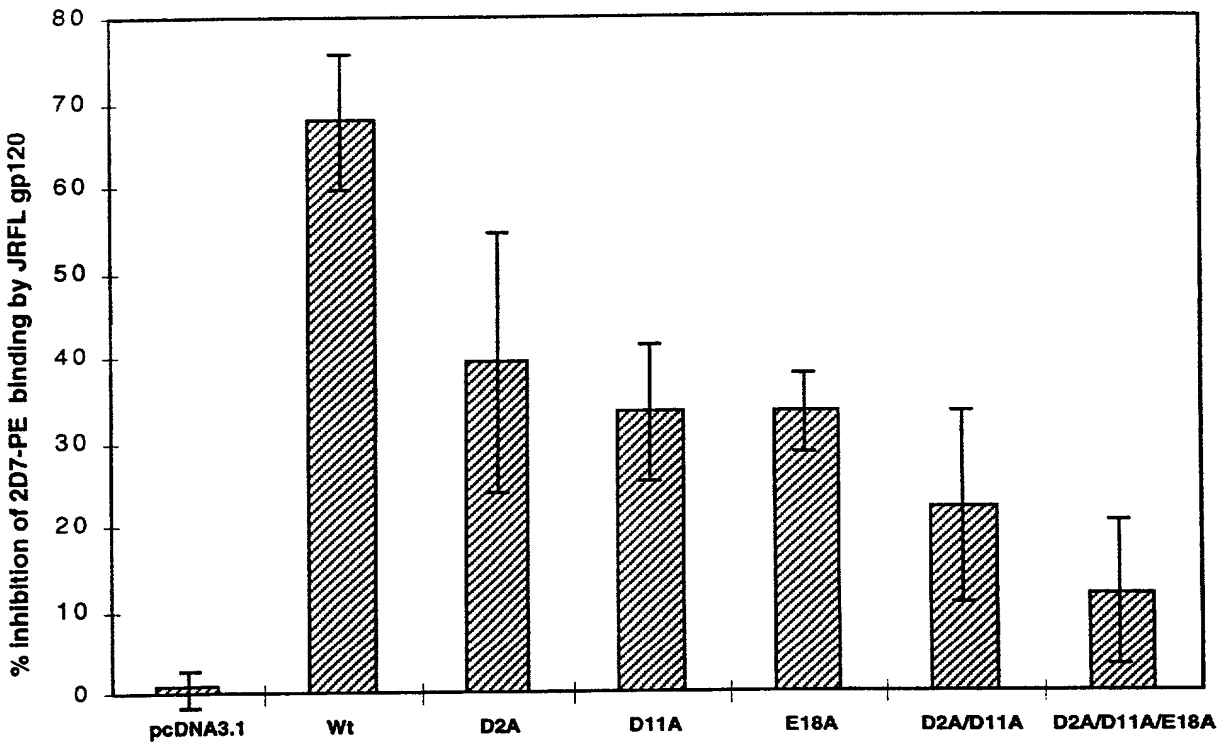

80
70
60
50
40
30
20
10
0
% Inhibition of 2D7-PE binding by JRFL gp120
pcDNA3.1
Wt
D2A
D11A
E18A
D2A/D11A
D2A/D11A/E18A

FIG. 8A

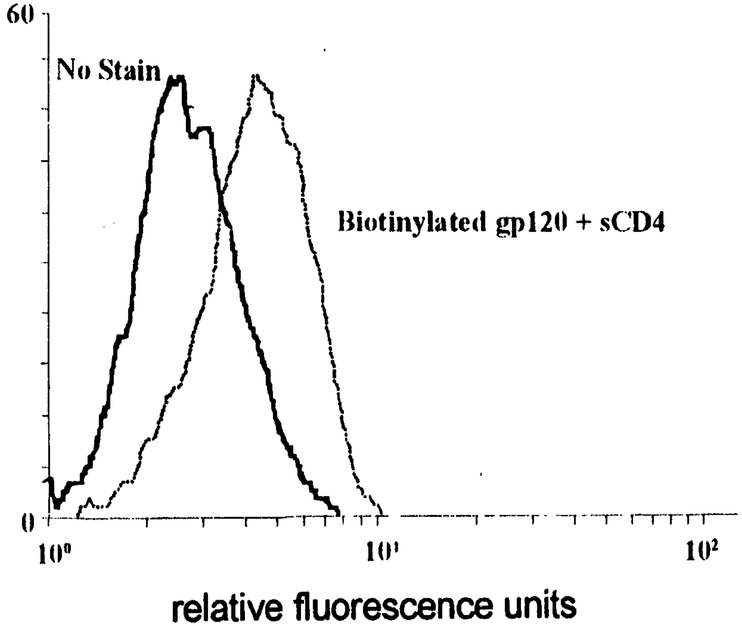

60
0
No Stain
Biotinylated gp120 + sCD4
10^0
10^1
10^2
relative fluorescence units

FIG. 8B

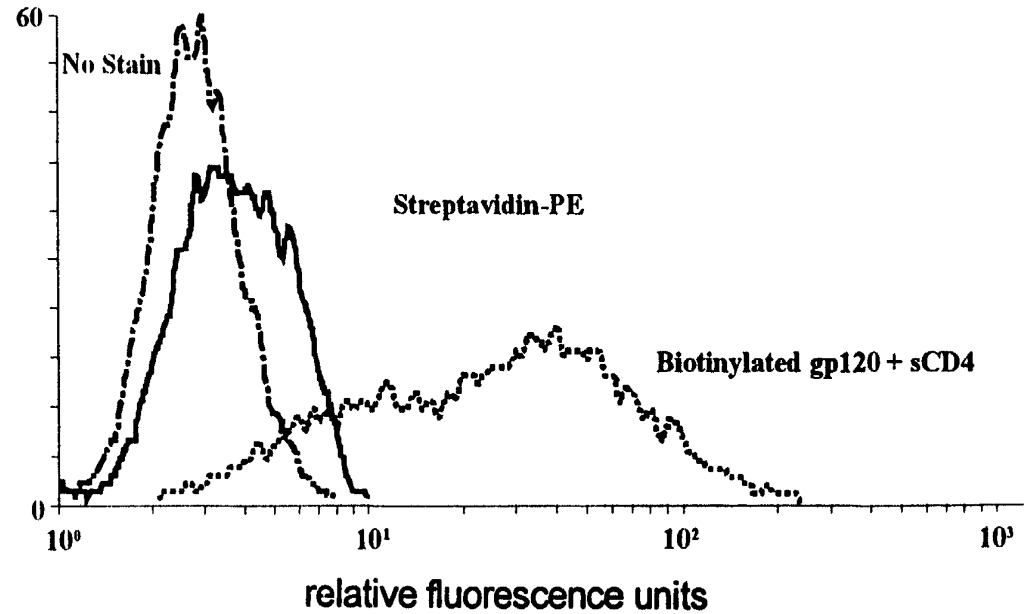

60
0
No Stain
Streptavidin-PE
Biotinylated gp120 + sCD4
10^0
10^1
10^2
10^3
relative fluorescence units

FIG. 9A

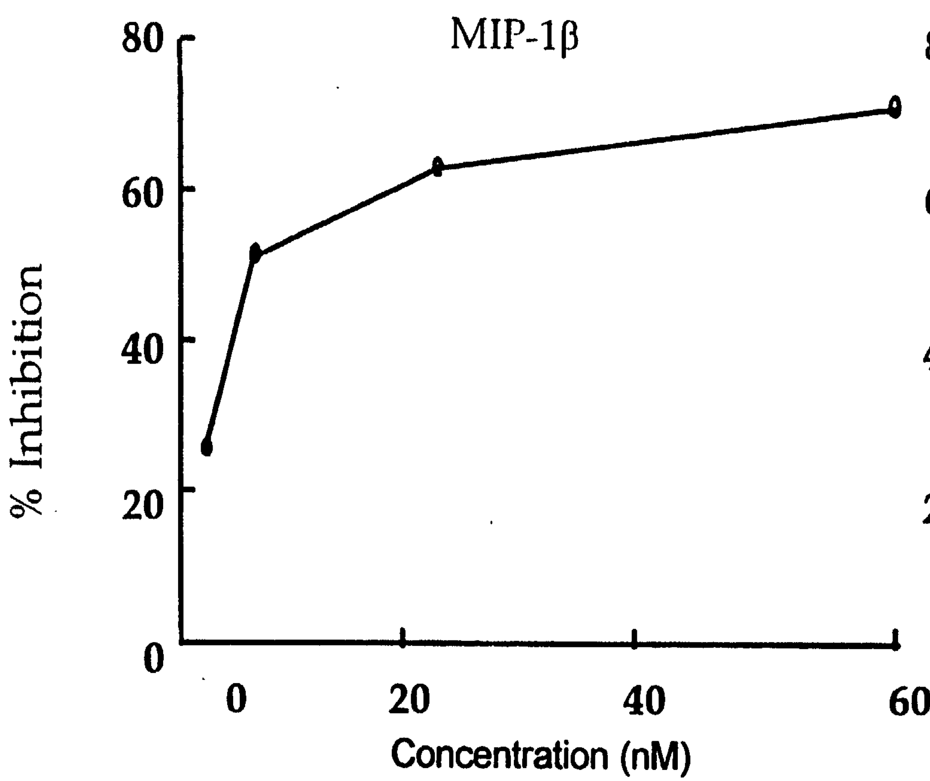

MIP-1β
% Inhibition
80
60
40
20
0
0
20
40
60
Concentration (nM)

FIG. 9B

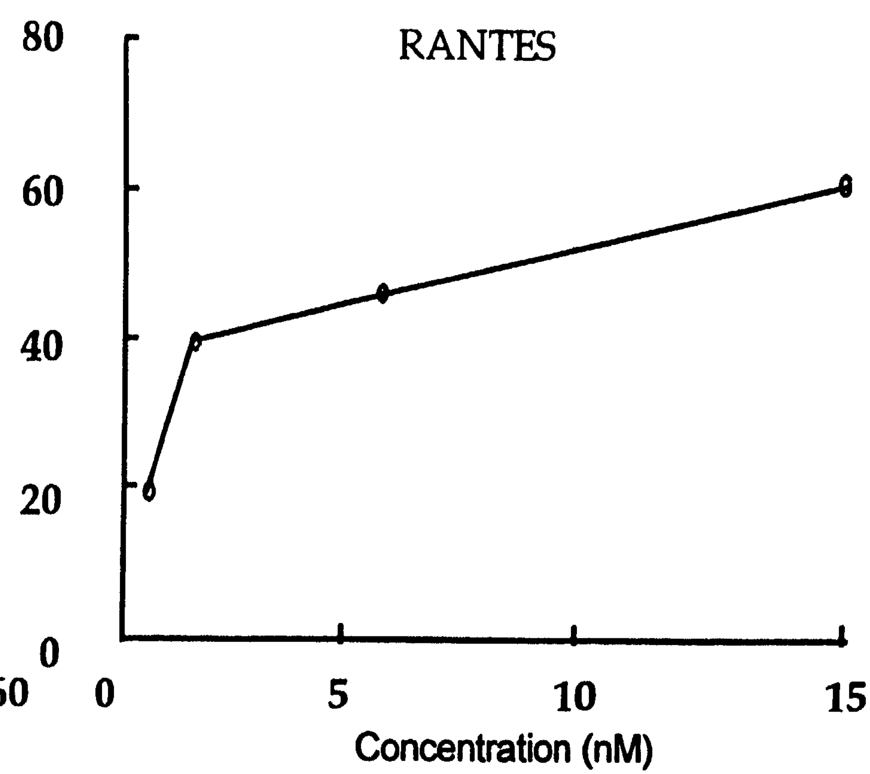
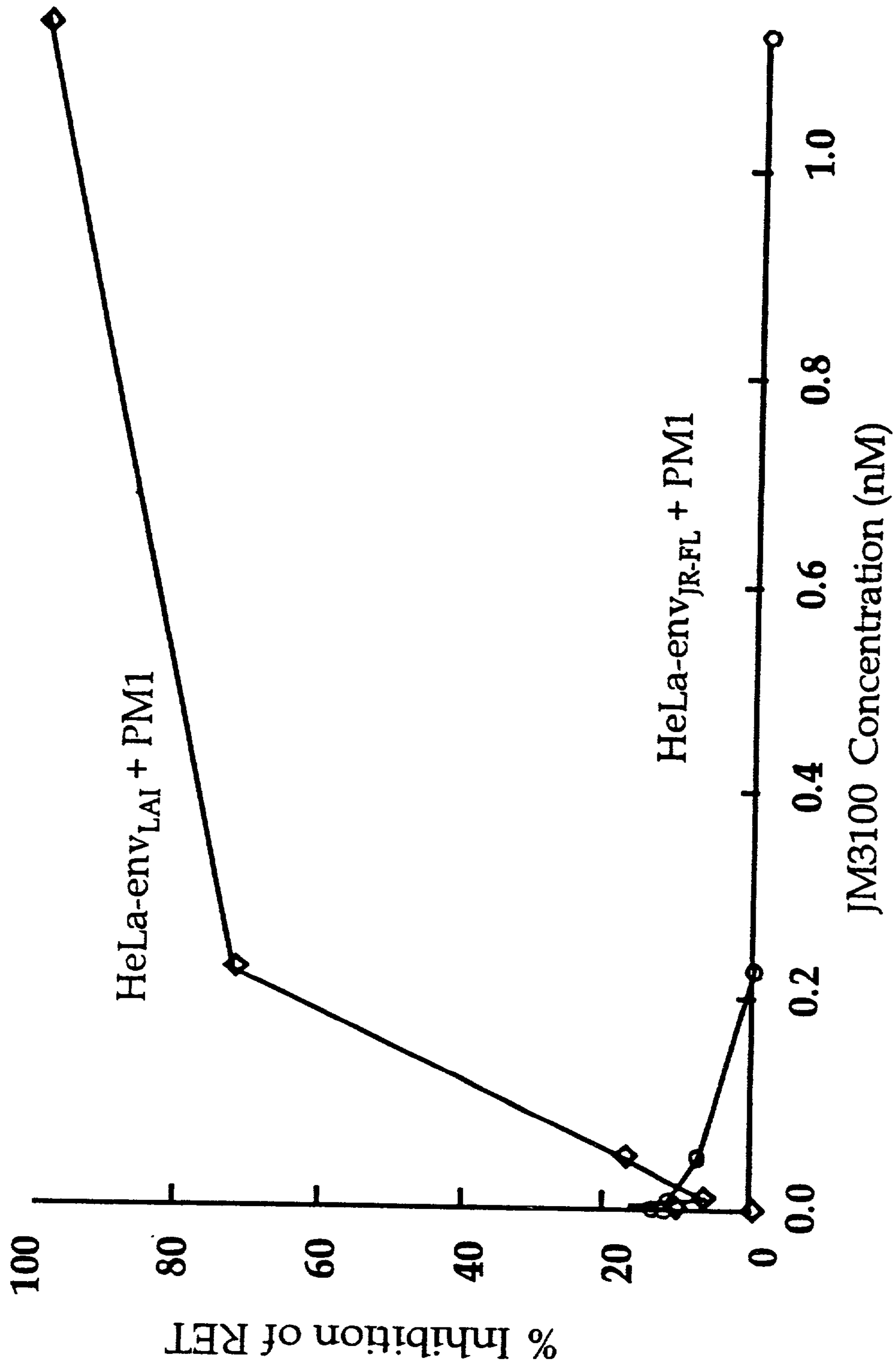

RANTES
80
60
40
20
0
0
5
10
15
Concentration (nM)

FIG. 10



HeLa-env$_{LAI}$ + PM1
HeLa-env$_{JR-FL}$ + PM1
% Inhibition of RET
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
JM3100 Concentration (nM)

Dkt. 51320-A/JPW/AKC

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

USES OF A CHEMOKINE RECEPTOR FOR INHIBITING HIV-1 INFECTION

the specification of which:
(check one)

___ *is attached hereto.*

X *was filed on* June 13, 1997 *as*

Application Serial No. 08/874,618

and was amended ______________.
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 (a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International Application which designated at least one country other than the United States, listed below. I have also identified below any foreign application for patent or inventor's certificate, or PCT International Application having a filing date before that of the earliest application from which priority is claimed:

Prior Foreign Application(s) *Priority Claimed*

Number	Country	Filing Date	Yes	No
N/A				

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

Provisional Application No.	*Filing Date*	*Status*
60/019,941	June 14, 1996	Pending

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s), or Section 365(c) of any PCT International Application(s) designating the United States listed below. Insofar as this application discloses and claims subject matter in addition to that disclosed in any such prior Application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56, which became available between the filing date(s) of such prior Application(s) and the national or PCT international filing date of this application:

Application Serial No.	*Filing Date*	*Status*
N/A		

And I hereby appoint

John P. White (Reg. No. 28,678); Thomas F. Moran (Reg. No. 16,579); Norman H. Zivin (Reg. No. 25,385); Ivan S. Kavrukov (Reg. No. 25,161); Christopher C. Dunham (Reg. No. 22,031); Thomas G. Carulli (Reg. No. 30,616); Robert D. Katz (Reg. No. 30,141); Peter J. Phillips (Reg. No. 29,691); Richard S. Milner (Reg. No. 33,970); Albert Wai-Kit Chan (Reg. No. 36,479); Mary Anne P. Tanner (Reg. No. 40,197); and Mary Catherine DiNunzio (Reg. No. 37,306)

and each of them, all c/o Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, New York 10036, my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to:

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Tel. (212) 278-0400

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true: and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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